Cellulases of *Thermoascus aurantiacus*

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欲窮千里目
更上一層樓

唐王之渙

To my late father,

Mum, Meng and members

of the family.

ABSTRACT

A β-glucosidase and three cellulolytic enzymes designated as cellulase I, II and III have been purified from culture filtrates of *Thermoascus aurantiacus*. The enzymes were homogeneous in disc-gel electrophoresis. Data obtained from gel filtration revealed their molecular weights to be 85,000 (β-glucosidase); 78,000 (cellulase I); 48,000 (cellulase II) and 34,000 (cellulase III): the carbohydrate content of these enzymes were 33.0, 5.5, 2.6 and 1.8% ($^{w}/_{w}$), respectively.

The β-glucosidase and the cellulases all showed a similar temperature optimum (approx. 70^{o}C) and pH optimum (pH 4.5 - 5.0). When incubated at 65^{o}C at their optimum pH for one hour, there was no loss of activity by any of these enzymes.

The three purified cellulases degraded native celluloses to different extents. Cellulase I was capable of acting on substrates with β-1,4 and mixed β-1,3; β-1,6 linkages. Cellulase II had no activity on soluble CMC but was particularly active in hydrolysing insoluble celluloses with cellobiose as the primary product. On the other hand, cellulase III degraded soluble CMC most readily with the production of cellobiose and other oligosaccharides as products. β-glucosidase was active against *p*-nitrophenyl-β-D-glucoside and released glucose from

cellobiose but had no hydrolysing ability on any form of cellulose. There was no synergism between the purified enzymes when tested on filter paper. The K_m determined are as follows: 1.0 mg/ml (β-glucosidase on *p*-nitrophenyl-β-D-glucoside); 8.8 mg/ml (cellulase I on CMC); 34.4 mg/ml (cellulase II on filter paper) and 4.6 mg/ml (cellulase III on CMC).

The mode of action of cellulase III on cellulodextrins and reduced cellulodextrins was examined. From the kinetic data and an analysis of the products, it is proposed that the specificity region of this enzyme is at least five glucose units in length.

These studies indicate that cellulase II is an exo-β-1,4-glucan cellobiosyl hydrolase; cellulase III, an exo-β-1,4-glucanase and β-glucosidase, a cellulase-free cellobiase.

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ABBREVIATIONS

CL	Chain length
CMC	carboxymethyl-cellulose
D.P.	degree of polymerisation
D.S.	degree of substitution
FP	filter paper
LODP	levelling-off degree of polymerisation
PAHBAH	*p*-hydroxybenzoic acid hydrazide
SDS	sodium dodecylsulphate
TEMED	N,N,N',N'-tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

Cellulose, the major component of all vegetation, is one of the world's most plentiful resources. Unlike other resources, such as oil and minerals, cellulose is constantly replenished by photosynthesis and growth of plants; it accounts for nearly one-half of the eighteen to twenty metric tons of organic carbon that is fixed by photosynthesis each year (Bellamy, 1969). It is a major component of (a) agricultural wastes (straw, stubble, leaves and stalks of many plants, rice and other hulls, peanut, almond, and other shells, corn cobs, bagasse); (b) food processing wastes (fruit peels, pulp, coffee grounds, pomace, vegetable trimmings); (c) wood wastes (brush, chips, bark, sawdust, paper mill fines); and (d) municipal wastes (40-60% of solid wastes chiefly as garbage and waste paper).

Biological degradation of cellulose is perhaps the biggest mass hydrolytic reaction taking place in nature, continuously contributing about 95 billion tons of carbon to the atmosphere annually. The enzymes that hydrolyse cellulose in nature play a major role in the carbon cycle and impose a considerable tax on the economics of the world by destroying useful cellulosic materials. However, a large number of rumen and soil microorganisms have been found to possess the capability of degrading native cellulose, and converting it into a wide range of products from hydrocarbons to carbohydrates. After many years of combating microbial degradation

of cellulose, investigators are now trying to accelerate the process. Owing to the food and energy shortages in the world, much interest of the research workers has been directed toward the search for hitherto unused renewable resources. The availability of cellulosic wastes ensures abundant cheap substrates for any processes that are developed. It is thus natural that a growing interest should be shown in the application of cellulolytic microbes and their enzymes to the utilisation of cellulosic materials. A prerequisite for all technical applications of this kind is a thorough knowledge of the enzymes and their properties as well as the selection of sufficiently active cellulolytic enzymes suitable for use in industrial processes.

1. Cellulose Chemistry

Cellulose is a long polymer of β-1,4 linked D-glucose residues. Evidence points to a structure of native cellulose in which more than 10,000 β-anhydroglucose residues are linked to form a long chain molecule. This means that the molecular weight of native cellulose is above 1.5 million. As the length of the anhydroglucose unit is 0.515 nm, the total length of the native cellulose is about 5 μm (Shitola & Neimo, 1975).

Native cellulose is an aggregate of well-defined partly crystalline microfibrils of indefinite length. Three different morphological models have been proposed (Figure 1).

(a) From early x-ray diffraction studies on cellulose, its partially crystalline structure was proposed to fit into a model called the fringe micelle which was for many years the widely accepted picture of cellulose crystallinity.

Cellulose could be described as an aggregate of individual glucose anhydride chains arranged more or less parallel to each other in the microfibril. This parallel arrangement occurs to a much lesser degree in amorphous and para-crystalline regions, whereas in crystallinic micelles (or crystallites) the chains are oriented strictly parallel by means of hydrogen bonds. Micelles measure at least 60 nm along the fibre axes and about 5 to 6 nm perpendicular to the axis; the cellulose molecules are believed to be long

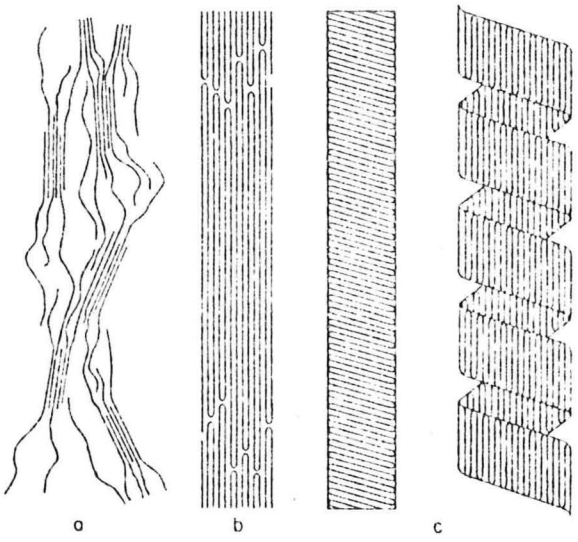


Figure 1. Morphological models of native cellulose

a. The fringe micelle. Models with folded cellulose chains; b. Folded along the fibre axis according to Marx-Figini & Schulz (1966a, b); c. Molecules forming a flat ribbon by folding back and forth perpendicular to the ribbon axis, the ribbon being wound into a helix according to Manley (1964) (after Marx-Figini & Schulz, 1966b, taken from Norkran, 1967).

enough to pass through several micelles. A cellulose fibre contains approximately 7.5×10^6 elementary fibrils, with relatively little space between (Mandels & Reese, 1965).

Since the concept of a folding process generally associated with the crystallisation of macromolecules is gaining ground (cf. Ranby & Noe, 1961; Davidovits, 1966), this idea has been adopted even for the molecular morphology of cellulose (models b and c).

(b) In Manley's (1964) planar zig-zag model the chain molecules form a flat ribbon by folding back and forth in a "concertina fashion", nearly perpendicular to the ribbon axis, the ribbon then being wound into a tight helix. Manley (1965) suggested four or five glucose units in the fold and hypothesised that hydrolysis of cellulose involves preferential attack at the chain folds.

(c) Marx-Figini & Schulz (1966a) suggested a flat ribbon formed by the molecule chain folded along the ribbon axis.

The possible molecular arrangements in the protofibril have been reviewed by Chang (1971). After a close look at the possibility of chain folding in cellulose and its effects, he discarded Manley's model and proposed a folded chain model of cellulose (Figure 2). According to his model, the cellulose molecule is folded back and forth in the plane parallel to the basic structural unit of cellulose. The distance between folds corresponding to the levelling-off degree of polymerisation (LODP). The crystallite consists of a number of platellites held together by secondary forces. Chain folding takes place through the occurrence of three of four successive glucosidic bonds which represent

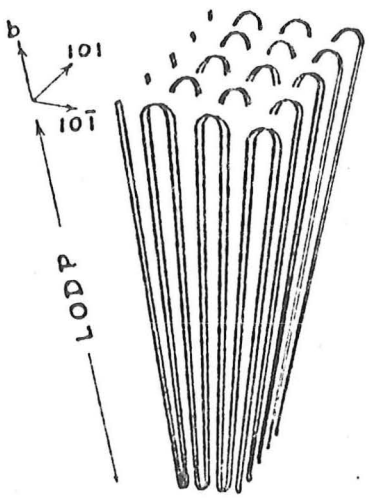


Figure 2. Perspective view of a cellulose crystallite according to the Chang model (after Chang, 1971)

weak points in the platellites and can be equated with the so called "amorphous" areas in the conventional terminology of cellulose structure. When discussing the probability for a folded cellulose molecule, Mühlethaler (1965) pointed out that a few molecules might be folded, but it is unlikely that all cellulose molecules are folded in the native material because many of the physical properties, for example, tensile strength, can only be explained if straight molecules are present. Thus Mark (1971) emphasised that certain mechanical criteria such as strength and elastic properties must be satisfied for any theory of cellulose structure to be consistent with experimental observations. These considerations refuted the concept of chain folding in native cellulose and, moreover, made the existence of amorphous cellulose unlikely, at least in the usually accepted sense. Fengal (1971) considered that a distinct loosening of the cellulose molecules in the sensitive regions was hardly

possible, as native cellulose is very resistant to enzymic hydrolysis, as isolated fibrils do not show such loosening points, and as they seem to be very stiff. Instead, he believes that slight deviations in the lattice order caused for example by the beginning of new chains, crossing-over or staggered arrangement of chains, may suffice for a higher sensibility to external influences in certain regions of the elementary fibrils. Another proposal was put forward by Viswanathan & Shenouda (1971) which assumes a helical chain conformation of native cellulose in which a complete turn of the helix requires seven cellobiose residues.

Whatever the molecular organisation of cellulose, it is apparent that a high degree of order exists in native cellulose. A consequence of this is that not even water molecules, not to mention enzymes, can enter the structure. Hence, native cellulose is very insoluble and very inert. Enzymic attack would be difficult even in the amorphous areas of cellulose and must be restricted largely to attack on loose chain ends and exposed surfaces. In crystalline areas the hydrogen bonds, as well as glucosidic bonds, must be ruptured for hydrolysis to proceed.

2. Cellulase Terminology

The term cellulase has been applied both to pure, well-characterised enzymes and to mixtures of enzymes produced by organisms which can degrade cellulose. These mixtures are sometimes called cellulase "complexes" or "systems". This implies a cooperative effect in the degradation of cellulose - not an integral, physical entity containing definite proportions of component enzymes.

Instead of the trivial name "cellulase", the Commission on Enzymes of the International Union of Biochemistry has assigned the systemic name β-1,4 glucan 4-glucanohydrolase (EC 3.2.1.4) which indicates a random or endo-β-1,4 glucanase activity with β-1,4 polymer of D-glucose as substrate. Exo-glucanases that act by removing either glucose or cellobiose units from the non-reducing end of the cellulose chain are designated as β-1,4 glucan glucohydrolase and β-1,4 glucan cellobiohydrolase respectively. The term "C_1" was first used by Reese *et al* (1950) for the enzyme that attacks the native cellulose initially. It was envisaged that only then could the subsequent action of the hydrolytic enzyme "Cx" take place. Recent work (Halliwell & Riaz, 1972; Wood & McCrae, 1972) have shown C_1 to be a cellobiohydrolase and the term "C_1" has now been replaced by exo-β-1,4 glucan cellobiohydrolase and "C_x" by endo-β-1,4 glucanase.

The trivial name cellobiase has been applied to widely different enzymes. With respect to cellulase enzyme components the term is applied here to an enzyme whose function is to degrade cellobiose produced during polymeric substrate degradation. Such an enzyme has been termed cellobiase by Selby & Maitland (1967), β-glucosidase by Wood (1968, 1971), and exo-glucanase by Li *et al.* (1965). The designation for β-glucosidases is EC 3.2.1.21. The terms hydrocellulase or avicelase refer to the type of substrate degraded by the enzyme, either alone or as a required component of a cellulase system.

3. Microbial Sources of Cellulases

The ability to produce cellulolytic enzymes is widespread amongst microorganisms. It is found among the gliding bacteria, among Gram-negative and Gram-positive true bacteria, and among actinomycetes (Goksøyr *et al.*, 1975; Siu, 1951). Cellulolytic ability is also found among obligate aerobes (*Pseudomonas*), facultative anaerobes (*Bacillus, Cellulomonas*) and obligate anaerobes (*Clostridium*). For the last decade or so most studies on cellulolytic enzymes have concentrated on a few fungi capable of degrading native cellulose. Such fungi are *Trichoderma viride, Trichoderma koningii* and *Trichoderma lignorum* (Wood & McCrae, 1972; Berghem and Pettersson, 1973; Eriksson & Pettersson, 1971; Mandels & Weber, 1969; Selby & Maitland, 1971; Mandels *et al.*, 1974; Griffin *et al.*, 1974 and Halliwell, 1975), *Sporotrichum pulverulentum* (Streamer *et al.*, 1975; Eriksson, 1975; Eriksson & Pettersson, 1975), *Penicillium funicolosum* (Selby, 1968a), *Penicillium iriensis* (Boretti *et al.*, 1972), *Myrothecium verrucaria* (Selby *et al.*, 1963), *Fusarium solani* (Wood & Phillips, 1969) and *Chaetomium thermophile var. dissitum* (Goksøyr *et al.*, 1975).

Thermophilic Fungi

In recent years interest has been shown in the activities of thermophilic microorganisms. Thermophilic fungi are abundant in self-heating piles of organic matter, including hay, wood chips, stored grains and mushroom composts and other agricultural composts (Chang, 1967; Hudson, 1967; Fergus, 1964 and Cooney & Emerson, 1964). They have caused much concern, both because they contribute

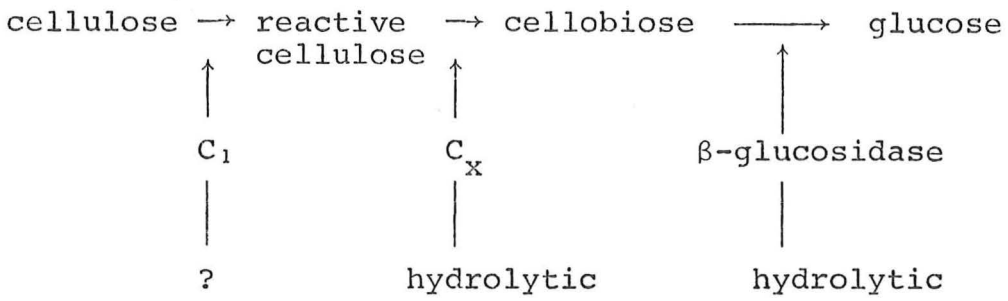
significantly to biodeterioration and spontaneous combustion of stored agricultural (Cooney & Emerson, 1964) and forest products (Tansey, 1971a) and because many thermophilic and thermotolerant fungi cause diseases of warm-blooded animals, including man (Cooney & Emerson, 1964; Pore & Larsh, 1967). The ability of thermophilic fungi to utilise cellulose has been studied by a few workers. Chang (1967) reported that *C. thermophile* and *Humicola insolens* were able to degrade filter paper to a large extent. In 1969 Fergus, working on the cellulolytic activity of thermophilic fungi and actinomycetes, observed that *C. thermophile var. coprophile*, *C. thermophile var. dissitum*. *Humicola grisea var. thermoidea*, *H. insolens*, *Myrothecium albomyces*, *Sporotrichum thermophile* and *Torula thermophila* were able to decompose filter paper and utilise soluble carboxymethyl-cellulose (CMC). Tansey (1971a,b) showed that cellulolytic activity of a number of thermophilic species was several times that of the most active cellulolytic mesophiles known. For example, *Thermoascus aurantiacus* dissolved acid swollen cellulose nearly three times as rapidly as *T. viride*. In 1975, Romanelli and co-workers reported that *S. thermophile* gave a higher rate of cellulose utilisation than *C. thermophile var. coprophile* and *T. aurantiacus*. Coutts & Smith (1976) extended the investigation of cellulases by *S. thermophile* and found yields comparable to those produced by *T. viride* in 14 to 18 days (Mandels & Weber, 1969; Mandels *et al.*, 1971) were obtained from *S. thermophile* in 3 to 4 days. It was concluded that one of the major differences between cellulases of *S. thermophile* and those of mesophilic fungi appears to be their rapid rate of production. Recently, Folan & Coughlan

(1978) showed that the culture filtrates of *Tararomyces emersonii* contain an active cellulase complex. These findings together with the studies on the occurrence and activities of thermophilic fungi in making mushroom compost (Fergus, 1964; 1971), in thermophilic composting as a means of waste disposal (Cooney & Emerson, 1964), in the production of industrially important enzymes (Barnett & Fergus, 1971; Somkuti & Babel, 1968; Somkuti *et al.*, 1969; Tansey, 1971b) as well as studies on thermostable enzymes and cellular components (Broad & Shepherd, 1970; Crisan, 1973; Miller & Shepherd, 1973; Miller *et al.*, 1974) emphasise the realised and potential value of thermophilic fungi in basic and applied research and fungi have been suggested to be the organisms most likely to be used for industrial production of cellulases (Enari & Markkanen, 1977).

4. Mode of Action

In 1950, Reese and his co-workers introduced their C_1 - C_x concept. This concept is based on the findings that some microorganisms are able to attack native cellulose while others are only able to degrade soluble cellulose derivatives such as CMC. Consequently, there appears to be some component present in the former systems that is missing in the latter. This component was designated as C_1 and was thought to be a chain-separating enzyme which at the time Siu (1951), as he said "in semi-jest", referred to as a "hydrogen bondase" whose presence was required before the hydrolytic enzymes (C_x) could start to break down the cellulose chain. Mandels & Reese (1964) described their C_1 - C_x concept in the following

schematic way:



They assumed C_1 "to act in a way to permit an increased moisture uptake, hydrating the cellulose and pushing apart the closely packed chains "to make the linkages accessible for the action of the hydrolytic β-1,4-endo-glucanase (C_x). To some extent, Siu's idea was supported by King (1966) and Liu & King (1967) when they reported fragmentation of hydrocellulose particles on exposure to *T. viride* cellulase. According to these workers, the C_1 component may not be an enzyme in the usual sense, but rather a protein, hydrogen bonding to cellulose more tightly than cellulose hydrogen bonds with itself. Such a process would cause the initial particles to gradually collapse and release ultimate micelles, thereby increasing the surface areas dramatically. Evidence that disaggregation caused by adsorption of C_1 is not an essential prerequisite for the degradation of cotton was put forward by Selby (1969). He reported the rate of adsorption of C_x-free C_1 on crystalline hydrocellulose is much more rapid than on cotton.

Ever since the C_1 - C_x concept was proposed almost three decades ago, the nature of the C_1 component of the cellulase complex has presented a challenging problem. The

existence of this component was strongly questioned some years ago by Whitaker (1953) who supported the "single enzyme theory" since cellulase isolated from *M. verrucaria* had only one component which was homogeneous by electrophoresis and ultracentrifugation. Some degree of activity towards cotton linter, CMC and cellobiose was exhibited by this enzyme. Such observation was also reported recently by Lobanok *et al.* (1976) with an enzyme from *T. lignorum*.

For many years much effort was directed towards seeking a solution to the mechanisms of action of cellulase in terms of either a uni- or a multi-enzymic system of C_1 and C_x components. The observation in 1964-1965 that culture filtrates from the cellulolytic fungi *T. viride* and *T. koningii* were capable of almost completely hydrolysing native cellulose (Mandels & Reese, 1964; Iwasaki *et al.*, 1964; Li *et al.*, 1965; Halliwell, 1965) was a turning point in the research on cellulolytic enzymes since they provided a possibility for isolating each of the separate enzymes. Some of the fractionation studies carried out on culture filtrates prepared from these two fungi (Mandels & Reese, 1964; Iwasaka *et al.*, 1964; Li *et al.*, 1965; Halliwell, 1965; Selby & Maitland, 1967; Wood, 1968) as well as *P. funiculosum* (Selby, 1968), *Polyporus versicolor* (Pettersson *et al.*, 1963), *Aspergillus niger* (Pettersson, 1963) and *F. solani* (Wood & Phillips, 1969) have shown them to contain at least three enzymes, or classes of enzymes, which are essential for the extensive hydrolysis of highly ordered forms of cellulose. Two of these types of enzymes have been arbitrarily called C_1 and C_x (Reese *et al.*, 1950), the third is a cellobiase or β-glucosidase. Enzymes classified as C_x can hydrolyse

swollen, soluble, or partially degraded cellulose, but are unable to attack highly ordered substrates such as the cotton fibre. C_1 component has little or no effect on either soluble derivatives of cellulose (such as CMC) or highly ordered substrates (Selby & Maitland, 1967; Wood, 1968, 1969, 1971; Wood & McCrae, 1972), but acts synergistically with C_x enzyme(s) and cellobiase to accomplish the conversion of native cellulose into water-soluble products (Mandels & Reese, 1964; Selby & Maitland, 1967; Wood, 1968, 1969). Yet a C_1 component capable, on its own, of solubilising crystalline hydrocellulose has also been observed (Li *et al.*, 1965; Flora, 1965; Ogawa & Toyama, 1967; Okada *et al.*, 1968; Olutiola, 1977) although it acts synergistically with the C_x components to bring about an increase in the rate of hydrolysis.

A different mechanism for cellulose degradation has been proposed by Leatherwood (1969) studying the cellulase complex of *Ruminococcus albus*. It was proposed that an "affinity factor" (C_1) and a "hydrolytic factor" (C_x) are necessary for the formation of a complete enzyme complex. To effectively hydrolyse insoluble cellulose, the hydrolytic factor must be held in position on the insoluble cellulose by the affinity factor.

Recently, work done by Halliwell & Riaz (1971), Halliwell & Griffin (1973), Wood & McCrae (1972), Berghem & Pettersson (1973), Berghem (1974), Eriksson & Pettersson (1975) concluded that C_1 is a cellobiohydrolase removing cellobiose units successively from the non-reducing end of the cellulose chain and Li *et al.* (1965) found that cellobiose was the sole product of hydrolysis of a crystalline hydro-

cellulose using their hydrocellulase (C_1). Eriksson (1969) challenged the original C_1 - C_x concept of Reese *et al.* (1950) by suggesting that the endo-1,4-glucanases (C_x), acting randomly over the cellulose chain, attack first and open up chains ends where the exo-enzyme (C_1) can act. This theory has been supported both by Wood & McCrae (1972) and by Berghem & Pettersson (1973). The synergism between the five endocellulases and the exocellulase of *S. pulverulentum* has been studied by Streamer *et al.* (1975). They showed that only a small amount of sugar (mainly cellobiose) was released by the exocellulase with cotton or Avicel as substrate. A pre-treatment of the substrate with endocellulase, however, increased sugar release considerably. On the other hand, a pre-treatment of the cotton with exocellulase had no effect on the amount of sugar released by the endocellulases. This observation clearly supports the theory of Eriksson (1969). An endocellulase with extensive cellulose powder and Avicel hydrolysing activity has recently been reported from *Irpex lacteus* (Kanda *et al.*, 1976a,b). Wood (1975) called for the C_1 - C_x concept to be abandoned and redefined the mechanism of cellulase action in new terms. These are, "that crystalline cellulose is effectively rendered soluble by the cooperative action of endoglucanase and exoglucanase enzymes: the exo-glucanase being of a special type that acts by removing cellobiose from the end of the cellulose chain". An example of such synergism was provided by the endo- and exo-glucanases of *T. viride* which Hofsten (1975) illustrated by means of models (Figure 3). Figure 3a illustrates a relatively simple substrate, cellobiose cleaved by β-glucosidase. Figure 3b illustrates the cleavage of a substituted cellulose derivative

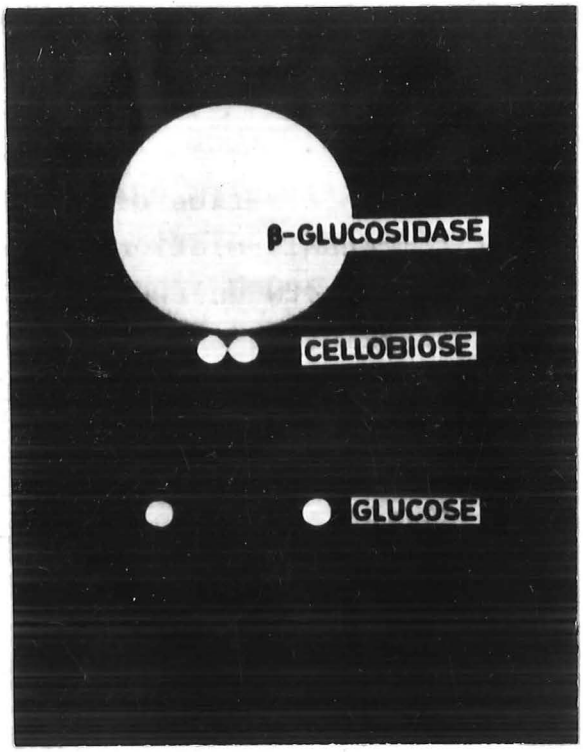
Figure 3 Models Illustrating the Synergism of *T. viride* Cellulases

(a) Model of a β-glucosidase molecule hydrolysing cellobiose.

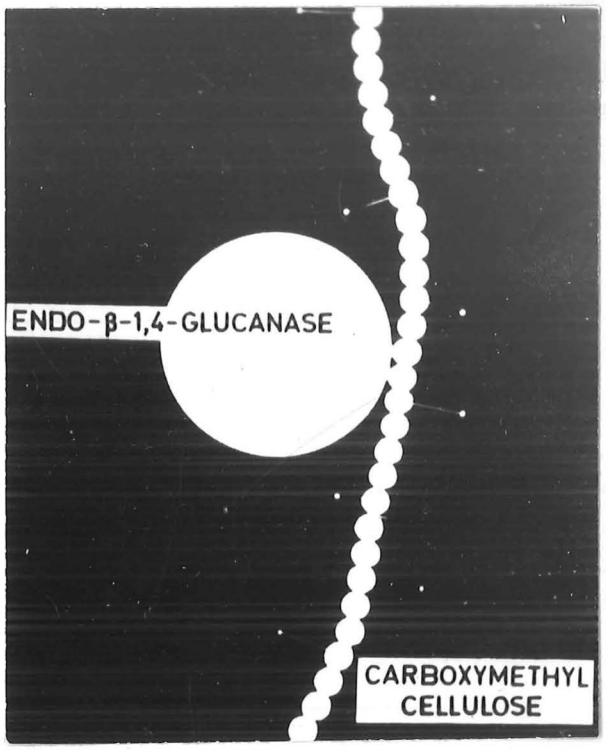
(b) Model of an endo-β-1,4-glucanase molecule on a carboxymethyl-cellulose chain.

(c) Model of a highly crystalline micro-fibril of the type presumably occuring in Avicel and an exo-β-1,4-glucanase hydrolysing cellobiose from the end of one of the forty glucosidic chains in the microfibril.

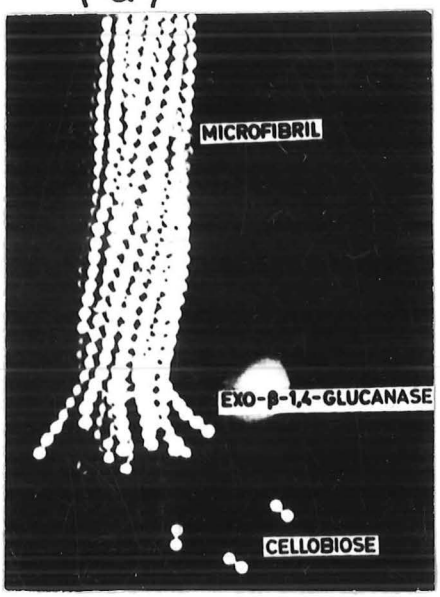
(d) Proposed mode of action of four different cellulolytic enzymes from *T. viride* on part of a cellulose fibre (After Hofsten, 1975).


β-GLUCOSIDASE
CELLOBIOSE
GLUCOSE

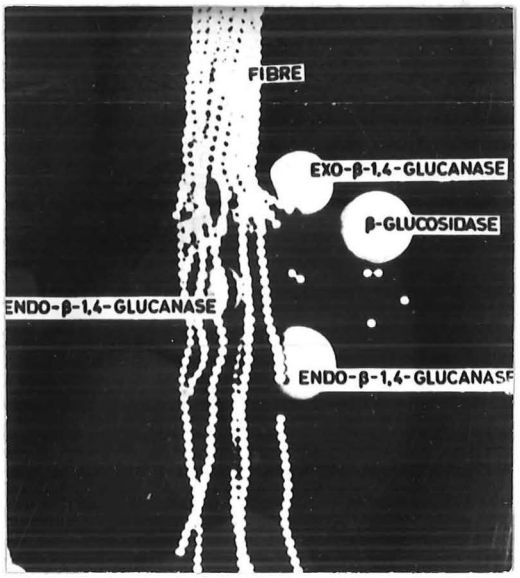
(a)


ENDO-β-1,4-GLUCANASE
CARBOXYMETHYL
CELLULOSE

(b)


MICROFIBRIL
EXO-β-1,4-GLUCANASE
CELLOBIOSE

(c)


FIBRE
EXO-β-1,4-GLUCANASE
β-GLUCOSIDASE
ENDO-β-1,4-GLUCANASE
ENDO-β-1,4-GLUCANASE

(d)

such as CMC by an endo-glucanase. Figure 3c shows a model of a crystalline fibrillar segment being degraded from its end by an exo-β-1,4-glucanase. Figure 3d illustrates degradation of part of the cellulose fibre as it may occur by the action of four of the different cellulolytic enzymes which have been isolated from *T. viride*. One of these is an endo-β-1,4-glucanase of very low molecular weight, 12,500, which might well penetrate into amorphous regions in fibres and break the glucosidic chains in them. The other endo-glucanase also contributes to the depolymerisation process, making it possible for the exo-glucanase to start its action in the manner illustrated in Figure 3c. Figure 3d also shows a β-glucosidase molecule cleaving cellobiose.

It has also been demonstrated that C_x from one fungal species can operate with C_1 from another (Wood, 1970; Selby, 1968b), at least when both fungi have the C_1 - C_x system. However, not all endo-glucanases (C_x) can co-operate with C_1. The explanation may be that the two enzymes, in order to achieve a synergistic effect, have to work together in the form of a loose complex. A slight shift in charge or tertiary structure could then prevent the formation of a cooperative system (Goksøyr *et al.*, 1975).

Reports of other manifestations of an initial attack on the cotton fibre have appeared in the literature, but probably the best known of these is the early change in cotton brought about by certain culture filtrates and subsequently measured as an increase in the uptake of alkali (so-called swelling-factor or s-factor activity) (Marsh *et al.*, 1953; Gilligan & Reese, 1954). Youatt (1962) and Wood (1968) have produced evidence supporting the original

suggestion of Galligan & Reese (1954) that s-factor activity is a property of one of the C_x-components and not of C_1. The fragmentation of cotton fibres to give short fibres, which has been reported by Halliwell (1965) is indicative of yet another change brought about before the appearance of significant quantities of sugar in solution. Halliwell & Riaz (1970) have shown, however, that short fibre forming activity is associated only with enzyme showing C_x activity. The loss in tensile strength of cotton fibres caused by "A" enzyme (Selby, 1963 Reese, 1963) has also been shown by Selby (1968b) to be more a property of C_x than C_1.

It is still possible that hitherto unknown enzymes are involved in the degradation of cellulose. The existence of one such enzyme was demonstrated by Eriksson *et al.*(1974). It was found that concentrated unfractionated culture solution of *S. pulverulentum* degraded 52.1% of de-waxed cotton, whereas the reconstituted solution degraded only 20%. When the culture solution was incubated in nitrogen instead of air, the degree of cellulose degradation decreased from the original 52.1% to 21.5%. This indicates that there is an additional oxidising enzyme involved in the degradation of cellulose. Eriksson has suggested that the probable mode of action of this oxidising enzyme comprises insertion of uronic acid moieties into the cellulose, thus breaking the hydrogen bonds between chains. This oxidative enzyme was found to be cellobiose: quinone oxidoreductase which participates in the degradation of cellulose in wood. This enzyme needs a quinone as a co-substrate (quinones are released from lignin) and therefore cannot function in the degradation of pure cellulose. No oxidative enzyme has so far been found to be

involved in extracellular cellulose degradation in the case of *T. viride* (Eriksson, 1978).

5. Properties of Cellulases

For the last decade or so, rigorous attention has been paid to the purification of cellulolytic enzymes using various chromatographic and electrophoretic techniques, thus making possible the studies of the physico-chemical properties of these enzymes.

The molecular weights of the exo- and endo-glucanases of *T. viride* (Selby & Maitland, 1967; Berghem *et al.*, 1976), *T. koningii* (Iwasaki *et al.*, 1965), *F. solani* (Wood, 1971), *Penicillium notatum* (Eriksson & Pettersson, 1968) and *P. funicolosum* (Selby, 1968b) were found to lie in the range 35,000 - 75,000, with the exception of the low molecular weight components from *T. koningii* and *T. viride*: these have a molecular weight of about 13,000 (Pettersson, 1975; Wood, 1975). The molecular weights of the five endo-glucanases isolated from *S. pulverulentum* vary between 28,300 and 37,500. Selby & Maitland (1965) fractionated the cellulolytic enzymes of *M. verrucaria* by gel filtration to give three major components, the smallest of which has a molecular weight of only 5,300. An even smaller molecular weight of 4,500 from *Botryodiplodia theobromae* has been described (Umezurike, 1970b) to be the subunit of all the other three cellulase components of higher molecular weight. This observation is based on the fact that reduction of the various components with 8M urea and 2-mercaptoethanol resulted in only one polypeptide subunit. Alternatively, it might be that this polypeptide sub-

unit is itself made of two smaller subunits each of molecular weight of about 2,200. Whitney *et al.*(1969) indeed found a cellulolytic component with a molecular weight of 2,200 in culture filtrates of *Verticillium albo-atrum*. These must be among the smallest proteins known to have enzymic activity. Pettersson & Porath (1963) separated the cellulolytic enzymes of *P. versicolor* by gel filtration and suggested that an inverse relationship might exist between the size of the enzyme components and that of the substrates towards which they are most active. Thus, low molecular weight cellulases show highest activity to highly polymerised cellulose whilst high molecular weight enzymes, with cellobiase as the extreme case, preferentially acting on cellobiose and oligo-saccharides. Although Wood (1971) described a cellobiase and CM-cellulase from *F. solani* with molecular weights of 400,000 and 37,000 respectively, the hypothesis may have little substance.

Another feature of cellulases is that they often form complexes with varying proportions of carbohydrates (Bjorndal & Eriksson, 1968). In extreme cases, a C_1 component of *S. pulverulentum* (Eriksson & Pettersson, 1975) with no carbohydrate and *T. viride* C_1 with 50% carbohydrate (Selby & Maitland, 1967) have been reported. The carbohydrate may be covalently linked to a protein moiety in some cases (Okada *et al.*, 1966; Eriksson & Pettersson, 1971) while present as dissociable complexes in others (Wood & Philips, 1969; Eriksson & Pettersson, 1968). Such a complex formation alters the physical properties of the enzymes and is found to account for the apparent heterogeneity of the CM-cellulase component in *Stereum sanguinolentum* (Eriksson & Pettersson, 1968) and

also in the β-glucosidase present in *Stachybotrys atra* filtrates (Jermyn, 1962). Both the major and minor components of the C_1 type of enzymes isolated from *T. koningii* by electrofocusing were shown to be isoenzymes: they differed in the extent to which they were associated with carbohydrate (9% and 33%). The carbohydrate content of the three CM-cellulases from *Chrysosporium lignorum* (Eriksson & Rzedowski, 1969) upon dialysis decreased to 13, 10 and 7 percent of the initial sugar content of the culture filtrates. All but one of the five endo-1,4-β glucanases (C_x) separated from *S. pulverulentum* (Eriksson & Pettersson, 1975) were glyco-proteins: the carbohydrate content varies between 0 and 10.5 percent. The carbohydrate moiety may consist of mannose and traces of galactose (Eriksson & Rzedowski, 1969) while others include glucose, glucosamine, xylose and arabinose as component sugars (Okada *et al.*, 1968; Eriksson & Pettersson, 1975; Kanda *et al.*, 1976a). However, it is difficult to imagine that varying amounts of carbohydrate could account for the differences seen in substrate specificity (Streamer *et al.*, 1975). Nakayama *et al.*(1976) have recently presented evidence that limited proteolysis may be responsible for the multiplicity of *T. viride* cellulases. The proteolytic modification was accompanied by minor changes in substrate specificity but a change in molecular size was not apparent.

The amino-acid composition of the enzymes often reveal a high content of acidic and aromatic amino acids and relatively few basic and sulphur containing amino acids (Pettersson & Eaker, 1968; King & Smith, 1974; Berghem *et al.*, 1975 and Hurst *et al.*, 1977). This accounts for their low isolectric points ranging from pH 3.3 (Ikeda *et al.*,

1973) to 5.74 (Berghem & Pettersson, 1974).

Thermostability is one of the most important properties of cellulases, since the hydrolysis of cellulose proceeds faster at higher temperatures. The optimum temperature may be 70°C for brief assays, although 50°C is the usual temperature for reactions of 1 to 24 h duration. For complete inactivitation of the enzymes, heating to 100°C for 10 to 20 min. may be necessary. The cellulase of *M. verrucaria* in the absence of substrate still had 20% of its original activity after heating for 10 min. at 100°C. The cellulase of *Rhizopus stolonifer* retained considerable activity after 10-15 min. of boiling. Endo-glucanases are more stable than exo-glucanases. Endo-glucanases are quite stable for up to 4 h at 60°C and pH 5.0. The β-glucosidase and exo-glucanase of *T. koningii* resemble one another in their heat stability at 60°C: they lost about 80% of their original activity at 60°C and pH 5.0 in 4 h (Wood, 1975). In the presence of cotton the cellulases of *T. koningii* and *F. solani* are remarkably stable, showing no loss of activity when incubated for 4 weeks at 37°C and pH 5.0 (Wood, 1975). However, not all of the cellulases have such excellent heat stability. It has been mentioned that exoenzymes of *A. niger* were quickly inactivated.

Fungal cellulases, in general, are stable from pH 3 to 8 at 30°C, active from 3.5 to 7.0, and usually show optimal activity at pH 4.0 to 5.5. The cellulase from *Pyricularia oryzae* (Hirayama *et al.*, 1978) shows a pH optimum of 5.5 and the cellulase of *T. koningii* have pH optima of 4.0 - 5.0 and 3.5 - 4.5 (Iwasaki *et al*, 1965). Double pH optima have also been reported for the cellulase

enzymes of *Aspergillus flavus* (Olutiola, 1976), *V. albo-atrum* (Whitney *et al.*, 1969) and *T. koningii* (Wood, 1968: Halliwell, 1965). A low pH optimum at 2.5 has been ascribed to a cellulase of *A. niger* (Ikeda *et al.*, 1973). Bacterial cellulases show higher pH optima, often around 7.0. For example, *Thermomonospora curvata* enzymes exhibit greatest activity at about pH 6.0 - 6.5 (Stutzenberger, 1971, 1972). Nematode cellulases have broad pH optima from 5.5 to 8.0 (Dropkin, 1963).

Inhibition of cellulases has been reviewed by Mandels & Reese (1965) and Norkrans (1967). Generally speaking, cellulases are inhibited by heavy metals, such as mercury, silver, copper, chromium, lead and zinc salts at about 10^{-3}M (Mandels & Reese, 1963). Oxidising agents are also strong inhibitors (Reese & Mandels, 1957). Inhibition by glucono-lactones and large organic molecules such as acidic or basic dyes, quaternary ammonium salts, or other detergents is reported to involve ionic binding and is affected by the pH of the reaction mixture (Basu & Whitaker, 1953; Pal & Basu, 1961; Reese & Mandels, 1957).

6. Applications of Cellulases

So far, cellulases have been limited to a few specific applications, but economic and ecological factors have increased interest in their potential value. In Japan, cellulases are produced commercially on a large scale. Most of the product is used in digestive tablets. For many years the Japanese have used microbial fermentation to upgrade grains and soybeans for use as human food (Underkofter, 1963). Today they are exploiting cellulases in a similar manner

(Yasumatsu *et al.*, 1966; Fujii & Toyama, 1964). It is claimed that nutritive value of food for animals is enhanced by such treatment (Moran, 1965). Fujii & Toyama (1967) found that treatment of rice with cellulase enzymes increases soaking efficiency and water absorption. An "instant rice" may be attainable in this way.

Valuable contents of plant cells (Toyama, 1963) can be released more effectively by use of enzymes. As long as 1962, cellulases have been proposed as a means of removing crude fibre from oil-seed press-cakes (Tazaki & Ouye, 1962), as a means of processing microbial cells for human food, and for extracting valuable cell contents from plant materials such as proteins from grass or soybean (Ramamott & Johat, 1963), agar from red algae (Toyama, 1962) and essential oils and flavours from various sources (Underkofter, 1963). Cellulases have been used in the clarification of citrus juice and in the production of stable juice concentrates. They have been incorporated into products promoting break-down in septic tanks, and they have been employed in paper-making to decrease the beating time of pulp and to dissolve the more readily digestable cellulose and hemicellulose constituents, leaving the more valuable fibres behind.

Disintegration of plant tissue has been experimentally provoked with cellulase preparations. Cocking (1960) succeeded in preparing protoplasts by removal of cell walls from root tips of tomato seedlings by means of a *Myrothecium* cellulase. Ruesink & Thimana (1966) improved the method partly by concentrating the cellulase and obtained proto-plasts showing vigorous cytoplasmic streaming. These plant protoplast are excellent experimental material for examining

a number of plant physiological problems such as wall formation, ion transport, and water balance. They have established the conditions for reproducible fusion of protoplasts from different species and have thereby opened a possible route for the production of hybrids from plants which cannot be crossed sexually. Thus, a new era in plant breeding is envisaged as cell lines are manipulated genetically by mutagens, protoplast fusion, or possibly transformed by DNA transfer (Emert *et al.*, 1974).

Recently, considerable effort has been expended in developing a commercially feasible process for converting waste cellulose to glucose. The economic advantages of enzymic over acid hydrolysis have been explained by Reese (1956) and Walseth (1952). Perhaps the most promising aspect of this process lies in the fact that it can help alleviate solid waste buildup, and, in the meantime, produces useful products. Municipal wastes, agricultural wastes and other cellulosic waste materials can be converted into sugars such as cellobiose and glucose by using cellulase enzyme. The importance of pre-treatment of the substrate and the use of more active enzyme preparations were realised. By treating a heated and milled cellulose with a concentrated *T. viride* cellulase, Katz & Reese (1968) obtained a glucose syrup of 30% concentration. Ghose (1969) and Ghose & Kostick (1969, 1970) studied this process in a complete multi-stage continuous system, using heated and milled cellulose, and culture filtrates of *T. viride*. In a continuous system with a 10% substrate concentration and a retention time of 40 h, effluents containing over 5% glucose were obtained. In subsequent studies (Ghose & Kostick, 1970) enzyme from a

mutant strain of *T. viride* was used. Using concentrated culture filtrate in a 30% suspension of milled cellulose (<25 μm), in a batch process, it was possible to obtain a solution of 14% glucose in 50 h. Application of the new ultrafiltration membranes made possible the development of a semi-continuous system, in which glucose passed through the sieves which retained enzyme and cellulose in the system. High rates of degradation were obtained, cellulose conversions approached 100% and the glucose was recovered as a clear aqueous syrup free of cellulose and protein. The freeze dried solids were over 80% reducing sugar. A continuous system to maintain the steady state condition was conceived (Ghose & Kostick, 1970). The sugars produced from these processes can be used as a source of glucose or they can be converted to protein, or to fat, by feeding them to the appropriate organism. The sugars could also be used for the production of alcohol. It has been suggested that internal combustion engines using alcohol would not pollute the atmosphere. Brazil has taken the lead in a programme for the mass production of ethanol by fermentation processes. Some corporations, such as TELESP, the telephone and telecommunications company of the State of Sao Paulo, are operating their automobile fleets on pure ethanol, with no gasoline added. It is obvious, therefore, that any increase in the yield of ethanol from sugar by fermentation would be of great significance, in a time when fossil sources of energy are dwindling. Respiration-deficient yeast mutants have been used in experiments aimed at improving the efficiency of ethanol production in the alcoholic fermentation of sugar-cane juice (Bacila & Horii, 1979).

The increasing need for protein and the availability of waste cellulose have focused much attention on the growth of cellulolytic microorganisms on waste cellulose to obtain "single cell protein" (SCP). Imre & Petch (1967) composted straw and hay for 21 days, pasteurised and inoculated with *Agaricus bisporus* (mushroom) which was allowed to grow for another 14-21 days. The resulting compost was found to be nutritionally equal to 175% of its weight of soybean protein and was proposed as a suitable fodder for various domestic animals. Srinivasan & Han (1969) reported direct conversion of bagasse (sugar cane residue) to *Cellulomonas* cells. This organism grows well on bagasse which has been milled and extracted with alkali and appears to produce a high quality protein. An 80% utilisation of bagasse with 23% dry weight protein content of the *Cellulomonas* residue was achieved. Development of a pilot plant to produce this bacterium in quantity sufficient for animal testing has been carried out. *M. verrucaria* has been proposed to produce protein from ball-milled newspaper; however, the final product contained no more than 10% protein (Updegraff, 1971). The highly bio-resistant lignin in newsprint, some 20-30% by weight, was responsible for the limited conversion of the newsprint to fungal cell mass. Fermentations with mixed cultures of *T. viride* and *Saccharomyces cerevisiae* or *Candida utilis* on treated barley straw resulted in a product of 22% protein (Pettersen, 1975). Studies on mesophilic and thermophilic microorganisms utilising cellulosic wastes by Bellamy (1974) have found that thermophilic organisms appear to be the most effective organisms for SCP production from waste cellulose. SCP production by thermophilic actinomycetes

from feedlot waste (cattle manure) resulted in a product of 30-35% protein. A fermentation carried out by *Thermomonospora fusca*, was developed which substantially degraded waste pulping fines, with the residual product of growth containing about 30% microbial protein (Crawford *et al.*, 1973). This protein, as shown by preliminary feeding study with baby chicks and by amino acid analysis, appears to be of good nutritional quality and contains no strongly toxic materials.

Hence, these studies are an attempt to develop processes for converting cellulosic wastes to a high-protein feed supplement for monogastric animals. The UNESCO report (1969) has stressed the importance of intensive research into the development of suitable processes for the production of SCP from cellulosic materials. Data for the commercial feasibility of the enzymatic hydrolysis of cellulose based on laboratory or pilot plant studies have been presented (Nystrom & Allen, 1976; Wilke & Yang, 1975). For economic evaluation of the process involved in the production of glucose or SCP, Das & Ghose (1973) reported that the estimated price of glucose or biomass from waste newspaper appears to be compatible with the reported market price of these products. Thus, waste cellulose and cellulases could become important commodities in an energy demanding world. The major problems encountered are obtaining sufficiently active cellulase enzymes and suitable substrates so that high concentration of glucose or SCP can be obtained in a reasonable period of time.

7. Aims of This Study

The potential value of thermophilic fungi in basic and applied research has been pointed out earlier. Yet thermophilic fungi have elicited very little investigation while thermophilic bacteria have been extensively studied. The need for further investigation of the degradation of cellulose by thermophilic fungi is apparent. Of the thermophilic fungi that have been studied in this regard, very few have been investigated in detail. Because of the fact that a complex of cellulase enzymes exists in fungi, it is necessary to examine the cellulase enzyme(s) of individual species to characterise the variability that may exist.

At the inception of this study, the degradation of cellulose by microbial enzymes including their identity and activity was far from clear. Thermophilic fungi possess obvious prolific cellulose degrading ability although this function has not been closely studied. The aim of this investigation was to examine the production of cellulase by a thermophilic fungus showing good enzyme activity. It was hoped to completely characterise the enzymes involved and in so doing clarify the nature of cellulose degradation.

CHAPTER II

MATERIALS AND METHODS

1. Fungi

(a) Isolation of thermophilic fungi

Samples collected from coastal beaches and compost heaps in Christchurch, New Zealand, were brought back to the laboratory in sterile McCartney bottles. Two isolation techniques were evaluated: direct hyphal isolation after the soil or the composting materials were incubated at elevated temperature to promote the growth of thermophiles; and direct inoculation method (Waksman & Gerretsen, 1931; Waksman *et al.*, 1939) in which samples were sprinkled lightly upon the surface of the media prior to incubation.

Thermophilic fungi were occasionally recognised right on the natural substratum and could be subcultured directly. Often, however, samples of the various organic substrata and soil did not reveal any thermophilic forms until they had been incubated. For this reason the organic substrata were chopped into small pieces and both the chopped materials and the soil well moistened, and placed in crystallising dishes that had previously been lined with several thicknesses of moist paper towelling. The dishes were then covered with glass lids and incubated at 50°C for several days until growth was established. The presence of contamination necessitated the isolation of individual hyphal

fragments before sufficient growth had occurred to permit their recognition. As a result, many duplicate cultures were isolated only to be discarded after the cultures were purified and the similarities recognised.

The hyphal isolates were subcultured onto fresh medium until pure cultures were obtained. The media used were Yeast-glucose (YG) agar and Yeast-starch (YpSs) agar.

The addition of 30 units of streptomycin per millilitre of medium provided a simple method of reducing bacterial contamination.

Standard sterile procedures were followed throughout culturing work.

Thermophilic fungus as defined by Cooney & Emerson (1964) is one that has a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or above 20°C.

(b) Choice of *Thermoascus aurantiacus*

By using the diagnostic key of Cooney & Emerson (1964), the isolated thermophilic fungi were identified as *C. thermophile var. coprophile*, *Humicola lanuginosa*, *Penicillium thermophile*, *S. thermophile* and *T. aurantiacus* (strain I). Another strain of *T. aurantiacus* (strain II) was isolated and provided by Dr A.L.J. Cole (Botany Department, University of Canterbury).

The cellulolytic ability of these fungi was tested (see Results) and it was found that culture filtrate obtained from *T. aurantiacus* (strain I) was the most active in breaking down filter paper and carboxymethyl-cellulose (CMC). Thus, this strain was chosen as the source of cellulase production for this work.

(c) Inoculum

The inoculum for each growth experiment was taken from cultures grown on YGA at 50°C for 48 h. An agar-mycelium disc (0.8 cm in diam.) was cut from the perimeter of the colony with a sterile cork borer. This disc was then inverted and placed at the centre of a petri dish containing the agar medium. In liquid culture experiments, one agar-medium disc (0.8 cm in diam.) was transferred to a Wheaton medical flat (C-16, 500 ml) containing 50 ml of the yeast-glucose medium.

For seeding liquid media, three agar-mycelium discs (0.8 cm in diam.) were placed into each of the Wheaton medical flats (C-16, 500 ml) containing 60 ml of Fergus's medium.

(d) Maintenance of stock culture

T. aurantiacus was routinely cultured on YGA slopes which were incubated at 50°C for 48 h and stored at 27°C for no more than 4 weeks prior to further culturing. For long-term storage, freeze-drying of whole culture was found to be effective.

2. Chemicals

(a) Enzyme Substrates

Whatman No. 1 filter paper and Whatman cellulose powder (CF 11) were obtained from Whatman Ltd, Sprinfield Mill, Maidstone, Kent ME 14 2LE, UK.

Carboxymethyl cellulose (CMC) type 7HF from Hercules, Wilmington, Delaware, USA.

Alkaline-swollen cellulose was prepared by a modifi-

cation of the method of Hash & King (1958) and the acid-swollen cellulose by the methods of Rautela & Cowling (1966) and Walseth (1952), as described later (see p. 33).

Solka-Floc SW-40 was a gift from the Brown Company, Berlin, New Hampshire, USA.

Acala cotton fibres and cotton yarn were kindly supplied by the Cotton Research Association, Shirley Institute, Didsbury, Manchester, UK.

Avicel micro-crystalline cellulose PH-101 was provided by FMC Export Corporation, Food and Pharmaceutical Products, Philadelphia, Pennsylvania, USA.

Yeast glucan, barley glucan and CM-pachyman were kindly supplied by Professor B.A. Stone, Department of Biochemistry, La Trobe University, Bundoora, Victoria, Australia.

Polyarabinogalactan was from Aldrich Chemical Company Incorporation, Milwaukee, Wisconsin 53233, USA.

Sodium polypectate was obtained from Sunkist Growers Inc., California, USA.

(b) General Chemicals

Ethylene glycol and ethanolamine were purchased from J.T. Baker Chemical Company, Phillipsburgh, NJ, USA.

Difco powdered yeast extract and Difco Bacto peptone were products of Difco Laboratories, Detroit , Michigan, USA.

Davis Bacteriological agar from Davis Gelatine Ltd, Christchurch, NZ.

Sephadex gels were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Bio-gel P-2 and P-60 were from Bio-Rad Laboratories, Richmond, California, USA.

Ampholine, pH 4-6 was from LKB Produkter AB, Bromma, Sweden.

Lactoferrin was purified as described by Baker & Rumball (1977).

p-Hydroxybenzoic acid hydrazide was purchased from Fluka, AG, Buchs, Switzerland.

Glucose oxidase, O-Diamisidine-HCl and cellobiose were products of British Drug Houses, Poole, Dorset, UK.

Glycerol was obtained from Merck, Darmsta'dt, Federal Republic of Germany.

Cellulodextrins and reduced cellulodextrins were prepared as described by Hurst *et al.*(1978).

2-Mercaptoethanol was bought from Koch-light Laboratories Ltd, Colnbrook-Bucks, UK.

All other biochemicals, standard proteins and enzyme substrates were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Wherever possible, inorganic chemicals were of analytical-reagent grade.

3. Preparation of Acid-swollen Cellulose

Fifteen grams of Whatman cellulose powder (CF 11) were placed into a beaker. 200 ml of 85% ($^{v}/_{v}$) ortho-phosphoric acid was slowly added and vigorously stirred with a glass rod to prevent formation of lumps. After 2 h, during which the mixture was constantly stirred, one litre of distilled water was added again with rapid stirring. This material was suction filtered through three layers of cheesecloth and two layers of Whatman No. 1 filter paper. The moist acid-treated products were combined in 2.5 litres of double-distilled water, suction filtered as before, then placed in 500 ml

of 2% (^{w}/v) sodium carbonate. After homogenising for 5 min at full speed in a Waring blender, this material was stored for 24 h. The preparation was then washed on a suction filter with 10 litres of distilled water and homogenised for 5 min. This yielded a suspension having a pH of 6.5. The total yield was calculated as 9.5 g based on dry weight of three one-ml samples of the suspension.

4. Preparation of Alkaline-swollen Cellulose

Cellulose powder (10 g) was dispersed in 100 ml 35% (^{w}/v) sodium hydroxide and left under vacuum for 30 min. The slurry was then poured into 900 ml of distilled water and stirred. The treated cellulose was suction filtered through Whatman No. 541 paper and washed with distilled water until free of alkali. The cellulose was dispersed in 10 ml sodium hydroxide/g rather than the recommended 6 ml/g because the latter was not sufficient to form a uniform slurry.

5. Determination of Cellulolytic Ability of Fungi

Initially, the organisms were grown in Reese-Mandels medium with CMC as carbon source. Each Wheaton flat (C-16, volume 500 ml) containing 60 ml of the liquid medium was inoculated with three agar-mycelium discs (0.8 cm in diam.). Incubation at 50^{o}C was for a period of 6 days. At the end of the incubation period, the liquid medium was suction filtered through glass fibre paper (Whatman GF/C) and the filtrate centrifuged at 10,000 g for 30 min. The clear supernatant was assayed for enzymic activity against CMC

by the viscometric technique (see p. 43).

Later, the organisms were grown in Fergus medium with filter paper as carbon source. After incubation at 50°C for 21 days, the liquid medium was treated as described above and the supernatant assayed for activities against filter paper and CMC (see p. 42,43). The cellulolytic activity in this case is expressed in terms of total reducing sugars (as glucose) produced, and is expressed as mg reducing sugar per ml.

6. Determination of Temperature Optimum for Growth

Optimum growth temperature was ascertained by measuring the diameter of colonies on YG agar medium in petri dishes containing 30 ml of the agar medium. Each agar plate was inoculated with one agar-mycelium disc (0.8 cm in diam.) as described earlier. Quadruplicate colonies were measured after incubation at temperatures of 30, 35, 40, 45, 50, 55 and 60°C ± 0.5 for 24 h and the average of the longest and shortest diameter of each colony was recorded. One 250 ml beaker of distilled water was placed in each incubator to reduce the dessication of the agar medium. Cultures incubated at 55 and 60°C were placed in polystyrene bags to further prevent dessication.

The dry mat weight of surface culture was also determined. Quadruplicate Wheaton flats (volume 500 ml) containing 60 ml of liquid yeast-glucose medium each were inoculated with one agar-mycelium disc (0.8 cm in diam.) and incubated at the temperatures noted above for 24 h. Mats were harvested by filtration using Whatman No. 1 filter paper and washed a few times with distilled water before drying to a constant weight at 60°C.

7. Effect of Temperature on Cellulase Production

Several Wheaton flats (C-16, 500 ml) containing 60 ml of the Fergus medium were inoculated and incubated at 30, 35, 40, 45, 50, 55 and 60°C ± 0.5. Two flats were removed from each temperature after the first, third and fifth day of incubation and then at regular intervals of 5 days. This was continued for 30 days. The contents were filtered and cellulase activities assayed on filter paper and CMC were determined by estimating the reducing sugars formed (see p. 40). The pH of the culture filtrate was also measured during each determination.

8. Preparation of Cell-free Culture Filtrate

Cultures of *T. aurantiacus* were grown in Fergus medium at 40°C for 21 days. The reddish brown contents of the bottle were suction filtered through glass fibre paper (Whatman GF/C) to remove most of the hyphal fragments and all of the residual insoluble cellulose. Reducing sugars and protein estimations of the filtrate were carried out and the volume recorded. The culture filtrate was concentrated by freezing the filtrate in Virtis flanged flasks (capacity 500 ml) and freeze dried using Virtis freeze-mobile (Model No. 10-147 MR-BA). 100 ml of citrate-phosphate buffer, pH 50 (0.1 M citric acid; 0.2 M di-basic sodium phosphate) was added to the dried material and stirred overnight at 4°C. Any undissolved substances were removed by centrifugation at 10,000 g for 30 min. at 4°C. The volume of the clear supernatant was made up to 100 ml (approximately 1/15th of the original volume of the crude culture filtrate) with

citrate-phosphate buffer and used as the source of cellulolytic enzymes. Purification of the enzymes was then carried out.

9. Column Chromatography

Unless otherwise stated, column chromatography was carried out at room temperature. Each gel was swollen in the appropriate elution buffer and packed according to the manufacturer's instructions. All columns were developed descending. An even flow rate was obtained by using a LKB 12000 Vario-Perpex pump. Fractions were collected automatically with an LKB 700 Ultrarac fraction collector. The protein content of the eluted fractions was recorded (LKB Typ 6520-4 recorder) by continuous measurement of the extinction at 280 nm and a portion of each fraction was examined for activity towards filter paper, CMC and *p*-nitrophenyl-β-D-glucoside. Active fractions corresponding to the enzyme activity were combined and concentrated by lyophilisation prior to the next purification step.

For the determination of molecular weights, a column of Bio-gel P-60 (2.2 x 47 cm) was calibrated by the technique described by Andrews (1964), using 0.05 M tris-HCl containing 0.1 M KCl, pH 7.5. Samples (2 - 6 mg) were applied in a volume of 1 ml and a constant flow rate of 15 ml/h was used for elution. Fractions of 2.5 ml were collected for analysis. Marker proteins were located by their absorption at 280 nm.

10. Polyacrylamide Gel Electrophoresis

Disc-gel electrophoresis was performed at 4°C by a modification of the method of Orstein & Davis (1964) in a 7.5% polyacrylamide get with bromophenol blue as tracking dye.

The composition of the gel was as follows: 18.3 g of acrylamide, 0.5 g of methylene-bis-acrylamide and 60 µl of Temed were dissolved in 225 ml citrate-phosphate buffer, pH 5.5 (0.1 M citric acid: 0.2 M di-basic sodium phosphate). 100 mg of ammonium persulphate was dissolved in 25 ml of the same buffer. A 7.5% gel was prepared by mixing the two solutions in the ratio 7.5:1, respectively. Glass tubes used were 0.5 x 9.0 cm. The buffer in both the upper and the lower tank was five times stronger than the buffer used for gel preparation. The gels were run initially at 3.0 mA per tube for 30 min. and then at 4.5 mA per tube for 8 h with the anode at the lower tank. The staining technique of Reisner *et al* (1975) was used.

For preparative purposes, several disc gels (0.7 x 12.0 cm) were run initially at 4.0 mA per tube for an hour and then increased to 7.0 mA per tube for a further 23 h.

Sodium dodecylsulphate (SDS) gels were performed and stained by the method of Weber *et al.* (1972). Gels were run initially at 4 mA/tube for one hour and then increased to 8 mA/tube for 13 h at room temperature.

11. Isoelectric Focusing

The analytical technique of Catsimpoolas (1968) was used for isoelectric focusing in 7.5% polyacrylamide gels.

Ampholines, pH 4 - 6 and later, 3.5 - 5.0, were used at an average concentration of 1% (^{v}/v). Electrofocusing at 4^{o}C was performed at a starting current of 5.3 mA per gel column (0.6 x 10.5 cm) which during electrofocusing dropped passively to 0.33 mA per gel. The isoelectric focusing was completed after 8 h at 350 v. The staining technique of Malik & Berrie (1972) was used.

Preparative isoelectric focusing was carried out as described by Vesterberg & Svensson (1966) in a LKB column with a capacity of 110 ml. The density gradient 0-60% (^{v}/v) ethylene glycol was formed using an automatic gradient mixer (LKB 8121). The concentration of carrier ampholytes, Ampholine, pH 4-6 was 1% (^{v}/v) and the electrodes were placed so that the anode was at the bottom of the column.

Five ml of 30-60% ammonium sulphate saturation fraction (approx. 17.5 mg protein) were electrofocused at a starting voltage of 600 v (16 mA) for 12 h and then reduced to a final voltage of 500 v (1.0 mA). After focusing for 66 h, the column was emptied at a rate of 84 ml/h. Fractions of approximately 1 ml were collected; their absorbance at 280 nm and pH were measured at room temperature.

12. Determination of Protein

Protein was determined by a modification (Eggstein & Kreutz, 1967) of the method of Lowry *et al.* (1951), with sodium citrate being used instead of sodium tartrate. Crystalline bovine serum albumin was used as standard. The absorbance at 280 nm was used for monitoring protein in column effluents.

13. Determination of Carbohydrate

Total carbohydrate was measured by the anthrone-sulphuric acid method of Herbert *et al*, (1971) using glucose as standard.

14. Determination of Reducing Sugars

Reducing sugars were estimated by the method of Nelson-Somogyi (Nelson, 1944; Somogyi, 1952). Copper reagent was prepared as follows: 24 g anhydrous sodium carbonate and 12 g potassium sodium tartrate were dissolved in 250 ml distilled water. Cupric sulphate (4 g) was dissolved in 40 ml of distilled water. The two solutions were combined slowly with stirring. Sodium hydrogen carbonate (16 g) was then added and stirred until dissolved. Anhydrous sodium sulphate (180 g) was dissolved in 500 ml distilled water and boiled. When cooled it was slowly added with stirring to the other solution and adjusted to a final volume of 1000 ml. The copper reagent thus made was stored at 35-40^{o}C and filtered after 3-4 days if necessary. Arsenomolybdate reagent was made by dissolving 50 g of ammonium molybdate in 900 ml of distilled water and added to 42 ml of concentrated sulphuric acid with stirring. Six g sodium arsenate in 50 ml distilled water were then added and the solution made up to 1000 ml. The reagent was stored in a dark bottle and was ready for use after standing for 24 h. To determine the amount of reducing sugars present, 0.5 ml of the solution containing reducing sugars was added to 1.0 ml of the copper reagent, mixed, and heated in a boiling water bath for 15 min. The samples were then cooled by

immersing in cold water and 1.0 ml of the arsenomolybdate reagent was added with immediate shaking until the colour developed completely. The volume was made up to 25 ml with distilled water, mixed thoroughly and the absorbance read at 560 nm using a Spectronic 20 spectrophotometer in earlier experiments and later on a Cecil CE 373 spectrophotometer.

In the studies of the mode of action against cellulodextrins and reduced cellulodextrins, a more sensitive assay based on the determination of reducing sugars released was carried out by the method of Lever (1973). *p*-Hydroxybenzoic acid hydrazide (PAHBAH) was prepared immediately before use. Five millilitres of the stock solutions: 1 M sodium sulphite, 0.2 M calcium chloride, 0.5 M *tri*-sodium citrate and 5 M sodium hydroxide were mixed and diluted to 90 ml with water; 1.0 g *p*-hydroxybenzoic acid hydrazide was dissoved in the solution which was then diluted to 100 ml. PAHBAH reagent (5 ml) was added to 0.6 ml solution containing reducing sugars, mixed and heated on a boiling water bath for 10 min. The samples were then cooled in cold water and the absorbance measured at 420 nm.

15. Enzyme Activity Measurements

Cellulase activity was measured by the appearance of reducing end groups liberated from filter paper or CMC. The number of reducing sugar groups created by hydrolysis of the cellulosic substrates was measured spectrophotometrically according to Somogyi (1952) using glucose as a standard.

An absolute definition of a unit of cellulase activity is difficult. This is because in the substrate such as CMC, the glucose molecules are substituted with carboxymethyl

groups, and the products of the enzyme reaction on filter paper and CMC are heterogeneous polymers; the effect of this on the absorption coefficient of reducing end groups is not known. It is not, therefore, valid to use a glucose standard to determine the number of μmol of reducing end groups. In addition, there is little to be gained by expressing the activity in terms of glucose equivalents, since glucose is not the only product of the enzyme reaction. A unit of each enzyme activity is defined below.

(a) Measurement of activity towards filter paper

An indication of total cellulolytic activity was obtained by the determination of filter paper degrading activity.

The standard reaction mixture containing 20 mg of filter paper (Whatman No. 1), 0.9 ml citrate-phosphate buffer (0.1 M citric acid, 0.2 M di-basic sodium phosphate) pH 5.0, 0.1 ml enzyme solution of appropriate dilution and one drop (10μl) of toluene was incubated at 60 C for 24 h. The mixture was then analysed for the production of reducing sugar. The amount of toluene added to prevent bacterial growth was found to have no effect on enzyme activity. Reaction mixtures were checked for contamination by withdrawing samples and streaking onto nutrient agar plates and incubated at 37 and 50^{o}C.

A unit of activity is defined as that amount of enzyme that produces an absorbance of 0.1 at 560 nm under the conditions defined.

(b) Measurement of carboxymethyl-cellulase activity

Viscometric method

In preliminary experiments designed to study the production of cellulolytic enzymes by the isolated thermophilic fungi, carboxymethyl-cellulase (CMC'ase) activity was assayed by a modification of the viscometric technique of Horton & Keen (1966).

CMC was dissolved in citrate phosphate buffer (0.1 M citric acid; 0.2 M di-basic sodium phosphate), pH 5.0 with Waring blender for 5 min. It was left in the cold room ($4^{o}C$) for 3 days and the viscous but clear supernatant phase was removed and the fibrillar matter at the bottom discarded. The concentration of CMC used was 0.75% ($^{w}/v$).

Five millilitres of the CMC solution in the viscometer (Cannon-Fenske, Type BS/lP/CF, size 300) and the diluted enzyme solution were equilibrated in a water bath at $50^{o}C$ for 5 mins. One ml of the enzyme solution was then added to the viscometer and mixed immediately. The reaction was timed from the moment of addition and the first measurement of the efflux time (number of seconds required for the meniscus to fall from the upper to the lower line of the viscometer) of the mixture was taken within 1 min. The determination of efflux times were continued on for 30 min. at 5 min. intervals. The percentage decrease in flow time (PDFT) after each incubation period (t) was calculated according to the formula,

$$PDFT_t = \frac{(E - E_t)}{(E - E_w)} \times 100$$

where E is the efflux time of CMC solution diluted with 1 ml buffer

E_t is the efflux time of CMC solution containing active enzyme after incubation for time t, and

E_w is the efflux time of distilled water in the same viscometer used for the above two determinations.

The $PDFT_t$ versus incubation time (including $PDFT_t = 0$ at zero incubation time) were plotted.

$$\text{Relative viscosity} = \frac{E_t}{E_s}$$

where E_s is the efflux time of the solvent, used for dissolving the substrate, in the same viscometer.

Specific viscosity = Relative viscosity - 1.

Reducing sugar assay method

In most of the work, cellulase activity was measured by the appearance of reducing end groups in a solution of CMC. The assay conditions were 0.9 ml of 0.75% ($^{w}/v$) CMC in citrate-phosphate buffer (0.1 M citric acid; 0.1 M di-basic sodium phosphate), pH 4.5 and 0.1 ml of enzyme solution incubated for 30 min. at 70°C. The rate of production of reducing sugars was determined. Any solubilisation that might occur under experimental conditions would be recognised in the substrate controls. A unit of activity is defined as that amount of enzyme that produces an absorbance of 0.10 at 560 nm under the conditions defined.

(c) Measurement of β-glucosidase activity

β-Glucosidase activity was assayed by a modification of the method of Umezurike (1969) using *p*-nitrophenyl-β-D-glucoside as substrate, 0.1 ml enzyme solution and 0.4 ml 0.001 M *p*-nitrophenyl glucoside in citrate phosphate buffer (0.1 M citric acid; 0.2 M di-basic sodium phosphate), pH 5.0, were incubated for 30 min. at 70°C. After incubation, 1.0 ml 1 M sodium carbonate solution was added to 0.5 ml of the assay mixture, diluted with 10 ml of distilled water and the nitrophenol released was estimated from the absorbance at 420 nm. One unit of β-glucosidase activity is defined as that amount of enzyme needed to liberate 1 µmol of *p*-nitrophenol per min. under the conditions of the assay.

(d) Measurement of cellobiase activity

Cellobiase activity was determined by measuring the release of glucose from a solution of cellobiose. To 0.5 ml of cellobiose solution (10 mg/ml) in the citrate-phosphate buffer, pH 5.0, 0.1 ml of enzyme solution was added. The mixture was then incubated at 70°C for 30 min. and the glucose released estimated by the glucose oxidase procedure of Lloyd and Whelan (1969). Blanks to test for the presence of glycosidases in the glucose oxidase were negative.

16. Paper Chromatrography

Products of cellulose hydrolysis were analysed by descending chromatography on Whatman No. 1 chromatography paper, in a n-butanol-acetic acid-water (120 : 30 : 50) solvent system. The samples were applied approximately 2.5 cm apart. After development for 22 h at room temperature, the paper

sheets were dried on air and sugars developed were detected with alkaline silver oxide reagent (Menzies & Seakins, 1969). The excess brown background was removed by immersing the paper in 10% ($^{w}/v$) sodium thiosulphate solution. Standard solutions of glucose, cellobiose and other oligosaccharides were prepared in 10% ($^{v}/v$) aqueous isopropanol and run as reference markers in parallel with the solution to be examined.

17. Kinetic Analysis

The enzyme kinetic parameters K_m and V_{max} were calculated by the graphical method of Lineweaver & Burk (1934) and also derived from data analysed by the direct linear plot (Eisenthal & Cornish-Bowden, 1974), using a programme developed for a Hewlett-Packard 9821A calculator. Velocities were expressed as units/µg protein and K_m values as mg substrate/ml or µmol *p*-nitrophenol in the case of β-glucosidase.

CHAPTER III

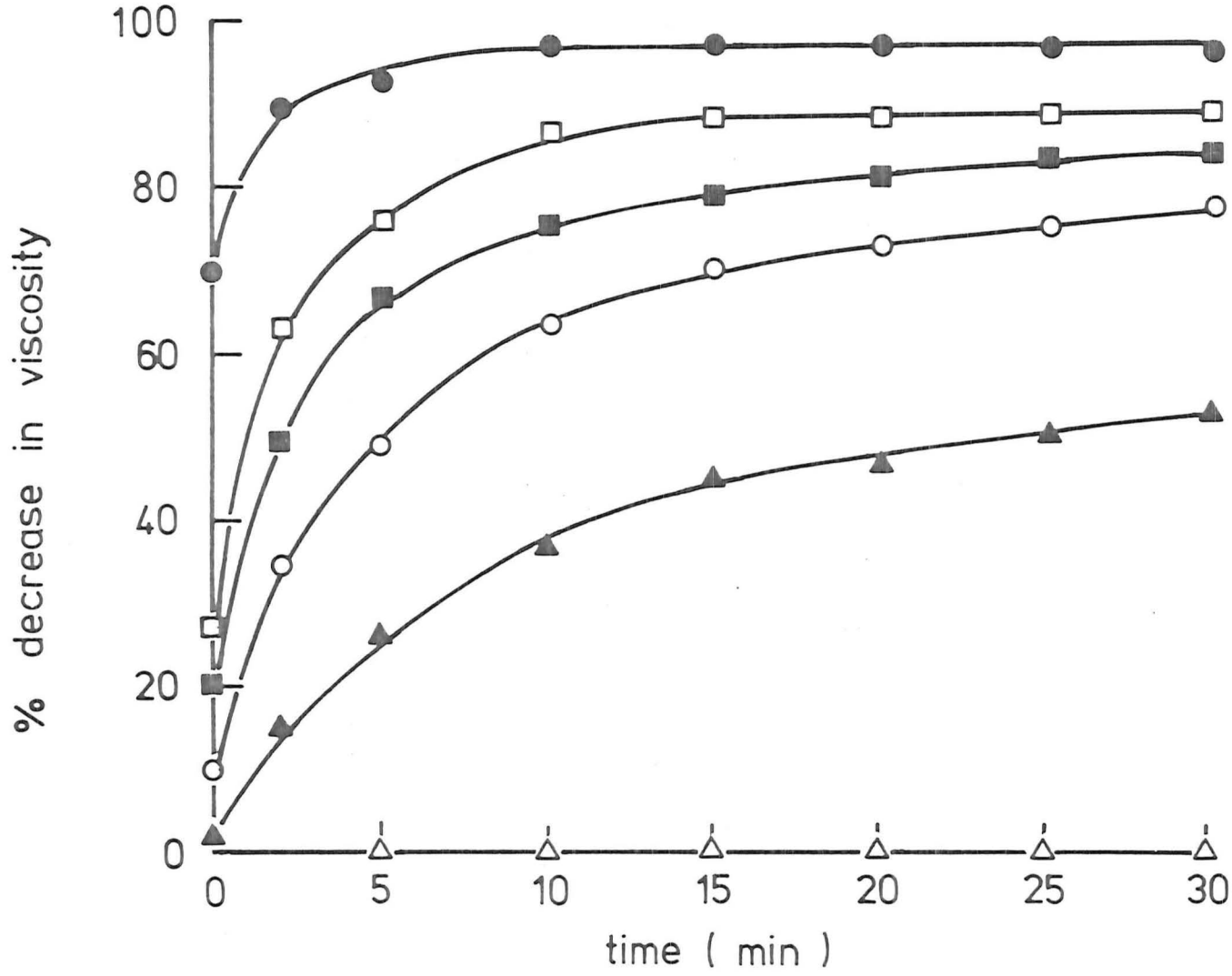
RESULTS

Comparison of Cellulolytic Ability of Isolated Thermophilic Fungi

Initial experiments were designed to discover which of the culture filtrates obtained from the isolated thermophilic fungi, namely *C. thermophile var. coprophile*, *H. lanuginosa*, *P. thermophile*, *S. thermophile* and *T. aurantiacus* (strain I and II) was most active in degrading soluble CMC, as well as insoluble forms of cellulose, such as filter paper. When grown in Reese-Mandels liquid medium (refer Appendix), all the cultures except *H. lanuginosa* and *P. thermophile* grew well on CMC as a main carbon source and the culture filtrates obtained caused a dramatic decrease in the viscosity of a CMC solution (Figure 4). *T. aurantiacus* (strain I) was found to be the most active cellulase producer. An investigation of the cellulase production by this strain over a period of 18 days revealed that maximum activity was reached after 2 days' incubation at 50 C, the enzyme activity then remained constant for as long as 18 days. *C. thermophile var. coprophile* and *S. thermophile* also hydrolysed the CMC. The cellulase activity produced by *P. thermophile* reached a maximum after 6 days' incubation and then declined steadily until there was no activity after 12 days incubation. *H. lanuginosa* could not utilise cellulose and no cellulolytic activity could be detected in the culture filtrate. The

Figure 4. Hydrolysis of CMC by Culture Filtrates of Thermophilic Fungi

5.0 ml of 0.75% CMC (7.5 mg/ml, in 0.1M citric acid; 0.2M di-basic sodium phosphate buffer, pH 5.0) and 1.0 ml culture filtrate were mixed and the efflux time of the mixture determined at the times indicated with a viscometer. The percentage decrease in the viscosity of the CMC solution after each incubation period was calculated as described in Materials and Methods. The experiment was carried out in a constant temperature water-bath at 50°C. The thermophilic fungi tested were: *T. aurantiacus* (strain I) (●); *T. aurantiacus* (strain II) (□); *C. thermophile* var. *coprophile* (■); *S. thermophile* (○); *P. thermophile* (▲); *H. lanuginosa* (△).


100
80
60
40
20
0
% decrease in viscosity
0
5
10
15
20
25
30
time (min)

slight growth of this fungus could be attributable to that allowed by the other organic constituents of the medium (1 g peptone and 0.1 g yeast extract per litre).

When these organisms were grown in Fergus medium (refer Appendix) with filter paper as carbon source, a similar pattern was observed for the degradation of cellulose. *H. lanuginosa* and *P. thermophile* failed to degrade filter paper. *C. thermophile* var. *coprophile*, *S. thermophile* and *T. aurantiacus* all grew well on filter paper. When the culture filtrates from these organisms were tested on filter paper, those of *T. aurantiacus* were shown to be the most active in degrading filter paper to reducing sugars. Strain I showed almost twice the activity of strain II (Table I). *C. thermophile* var. *coprophile* and *S. thermophile* also degraded filter paper to a moderate degree. Neither *H. lanuginosa* nor *P. thermophile* could break down filter paper.

TABLE 1 Degradation of filter paper by culture filtrates of thermophilic fungi

Organism	mg reducing sugar/ml
T. aurantiacus (strain I)	0.22
T. aurantiacus (strain II)	0.10
C. thermophile var.*coprophile*	0.04
S. thermophile	0.08
P. thermophile	0
H. lanuginosa	0

Reaction mixtures contained: 20 mg filter paper in 0.9 ml citrate-phosphate (0.1 M citric acid; 0.2 M di-basic sodium phosphate) buffer pH 5.0, and 0.1 ml cell-free culture filtrate. One drop (10 µl) of toluene was added and the mixtures incubated at 50°C for 24 h. Reducing sugars produced were estimated as described in Materials and Methods

Based on the results obtained from these experiments, *T. aurantiacus* (strain I) was chosen as the source of cellulase production for this work.

General growth characteristics of *T. aurantiacus*

The morphology and culture characteristics of *T. aurantiacus* grown in pure culture at 37°C on YG agar or oatmeal agar were described by Cooney & Emerson (1964). The general growth characteristics on YG agar of the isolated *T. aurantiacus* at two temperatures (37 and 50°C) agreed with those reported.

At 50°C, the growth of the vegetative mycelium was rapid, reaching the edge of a 9 cm petri dish from the central disc inoculum in less than 48 h. The growth at this stage was mainly confined to the surface of the agar and within the substratum. Later, the hyphae extended upwards from the basal mycelium, often reaching the petri dish lid. The colour of the culture was white. Microscopic examination of this culture is shown in Figure 6.

The chlamydophores were septate and irregularly branched. Long chains of conidia on the phialides as reported by Cooney & Emerson (1964) were not observed in this strain. Most of the chlamydospores measured about 6 x 2 µm and were formed singly on chlamydophores branching out of the hyphae as shown in Figure 6d. This chlamydospore stage was very delicate and evanescent. Complete collapse of the aerial system occurred merely by opening the lid of the petri dish.

At 40°C, the growth characteristics of the organism were different. The initial growth of the mycelium was

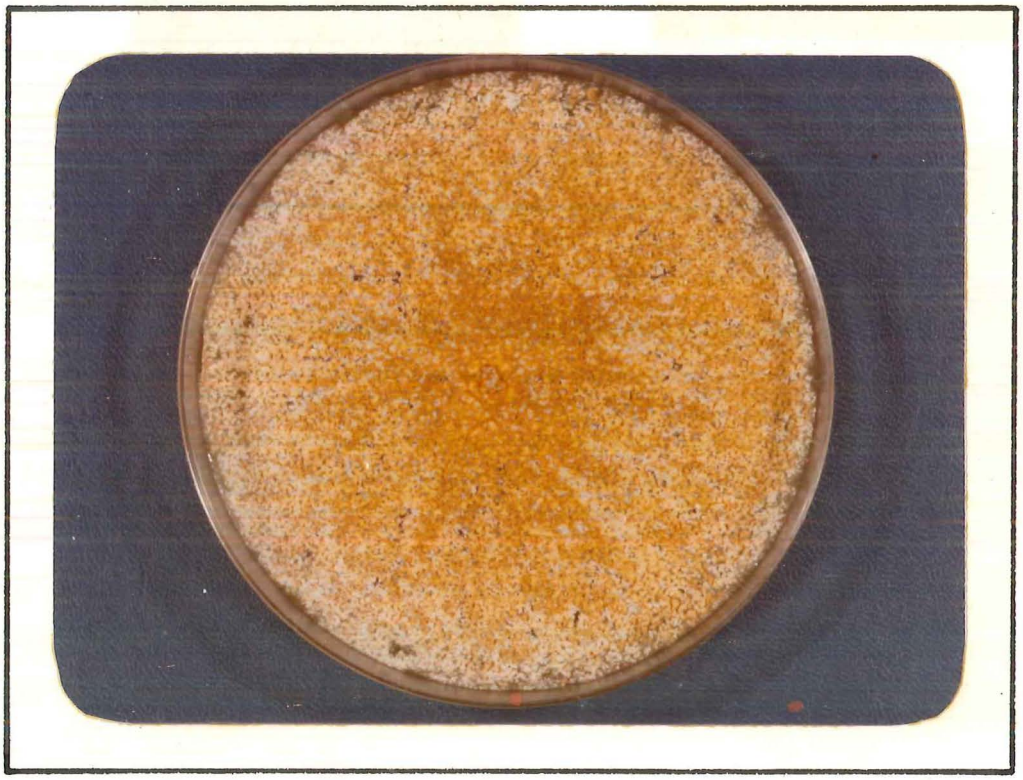


Figure 5. *Thermoascus aurantiacus*, 5 days at 40°C on yeast-glucose (YG) agar.

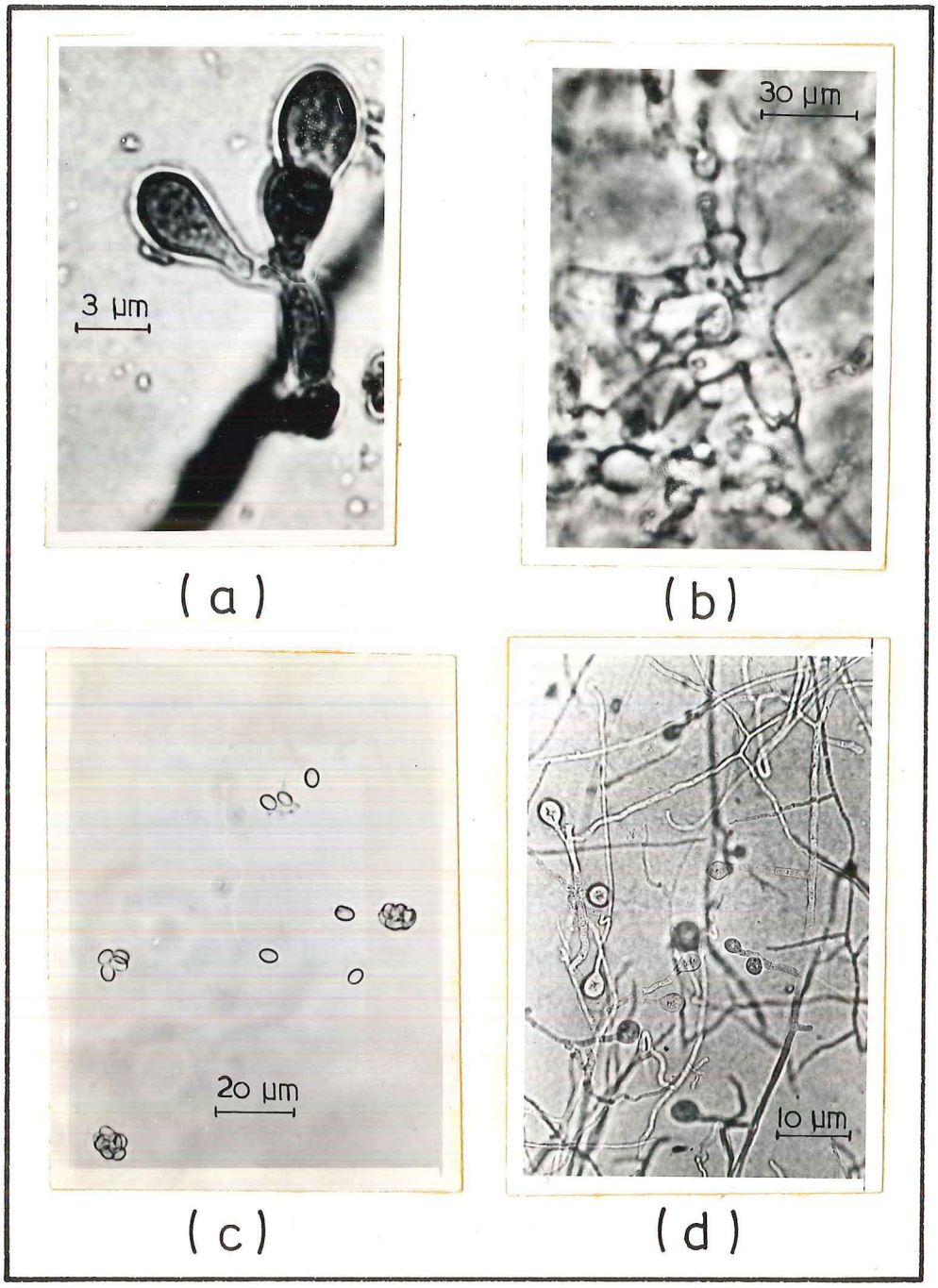


Figure 6. *Thermoascus aurantiacus.*

(a) Chlamydophore bearing a cluster of three chlamydospores.

(b) Large pseudoparenchymatous cells forming the outer peridial layer of the cleistothecium.

(c) Asci with maturing ascospores.

(d) Long branched chlamydophore bearing single chlamydospore at its apex.

slower. As early as the second or third day, cleistothecial initials became evident as small granular masses of knotted hyphae appeared in the white surface of the mycelium which then began to take on a yellow hue. As the pigment concentration increased the colour changed to orange-buff (Figure 5), bright brick-red, and finally a dull reddish-brown colour. At the same time, the underside of the mycelium followed the same pattern of pigmentation. Glistening drops of golden liquid were formed on the upper surface of the culture. All of these changes in pigmentation were associated with the development of ascocarps. Figure 6c shows asci measuring about 5 µm in diameter containing maturing ascospores. The outer peridial wall of the cleistothecium was composed of several layers of irregularly swollen, pigmented, pseudoparenchymatous cells varying considerably in dimension but were often as much as 15 µm across (Figure 6b).

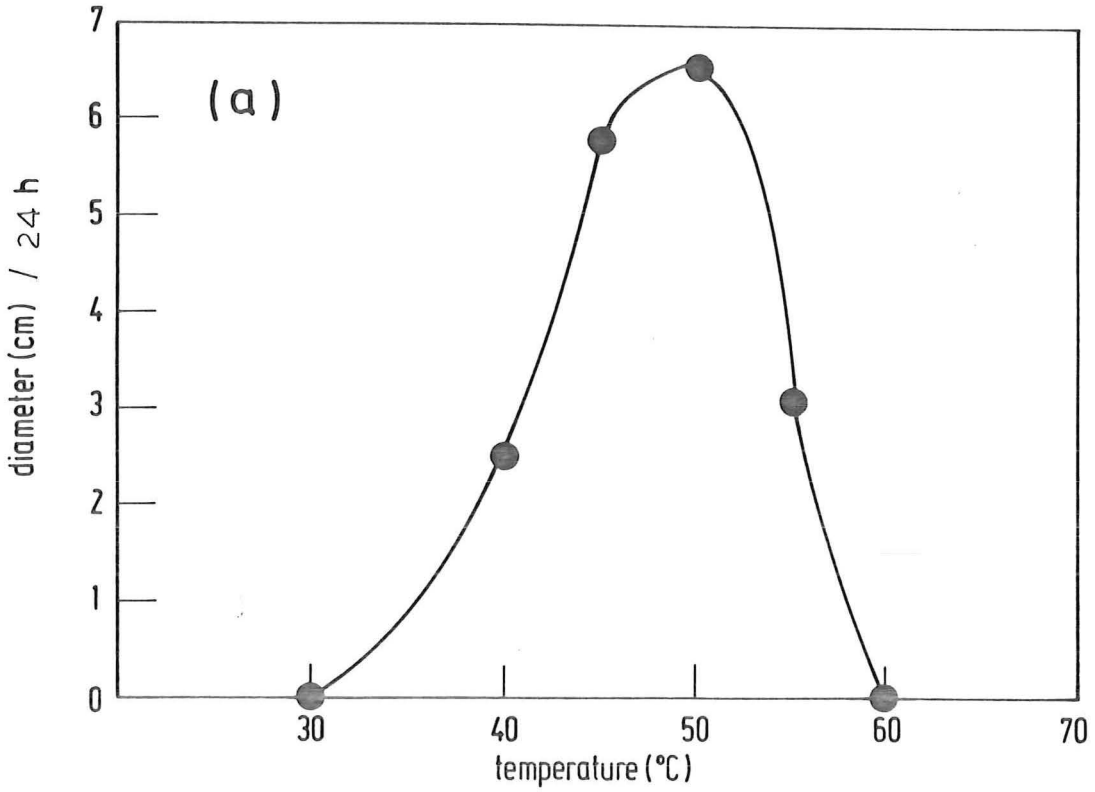
Temperature optimum for growth

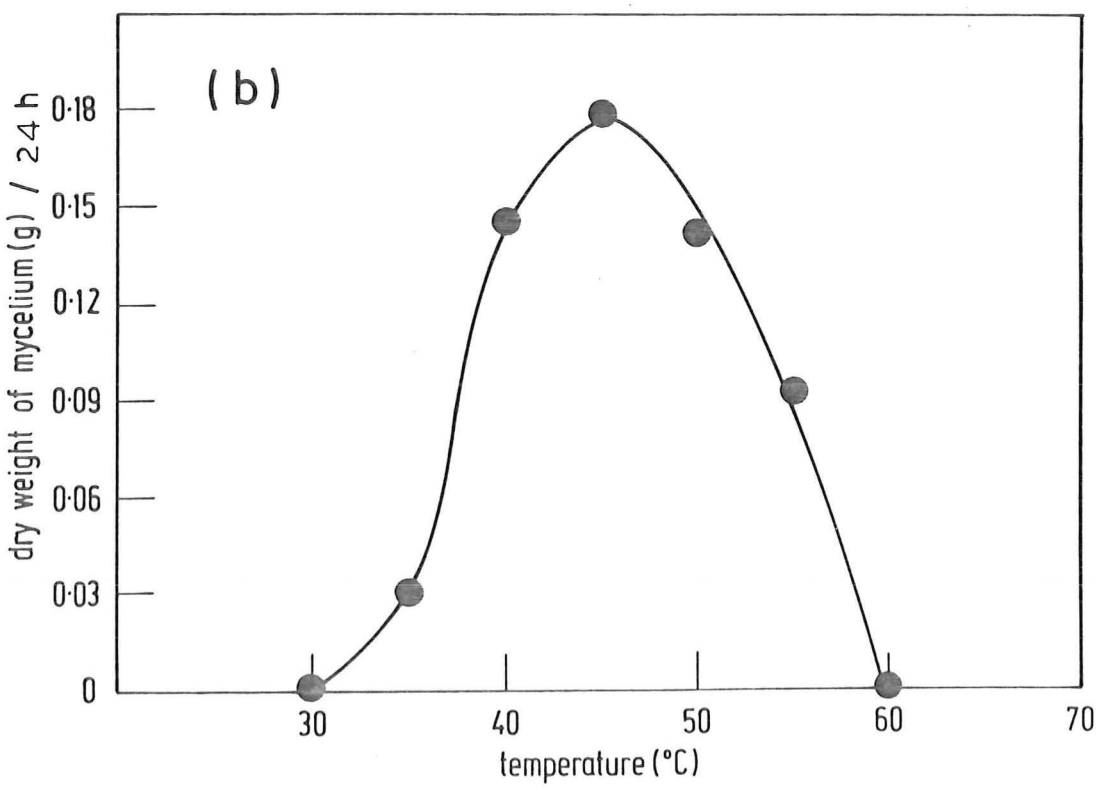
The growth of *T. aurantiacus* on YG agar and YG liquid medium was determined at the temperatures indicated in Figure 7. The criterion used for growth in solid medium was the increase in the diameter of the colony on the surface of the agar. In liquid medium, growth was determined by the increase in dry weight of the mycelial mat. The short incubation period (24 h) was necessary because of difficulties encountered at higher temperatures of the agar medium drying out and in keeping the liquid culture volume constant.

Results obtained with the diametric method of estimating growth are plotted in Figure 7a and can be compared with

Figure 7 (a). Diameter of *T. aurantiacus* Colony on YG agar at Different Temperatures.
Each point represents the average of two colony diameters in each of four replicates.

(b) Growth in YG Liquid Medium of *T. aurantiacus* at Different Temperatures.
Each point represents the average of four replicates.


(a)
diameter (cm) / 24 h
0
1
2
3
4
5
6
7
30
40
50
60
70
temperature (°C)


(b)
dry weight of mycelium (g) / 24 h
0
0·03
0·06
0·09
0·12
0·15
0·18
30
40
50
60
70
temperature (°C)

the mycelial weights in Figure 7b. The optimum temperature for growth on solid medium occurred at 46-51°C. In liquid medium the optimum was lower, at 45°C. No growth appeared at 30°C on either media. At 55°C, growth was retarded and ceased at 60°C.

The minimum temperature for growth cannot be accurately determined from the data presented due to the short growth period used. The fungus produced 0.1 - 0.2 cm of hyphal extension after one week at 30°C. However, there was no growth of the fungus at 60°C even after a prolonged incubation period, indicating a maximum temperature for growth between 55 - 60°C.

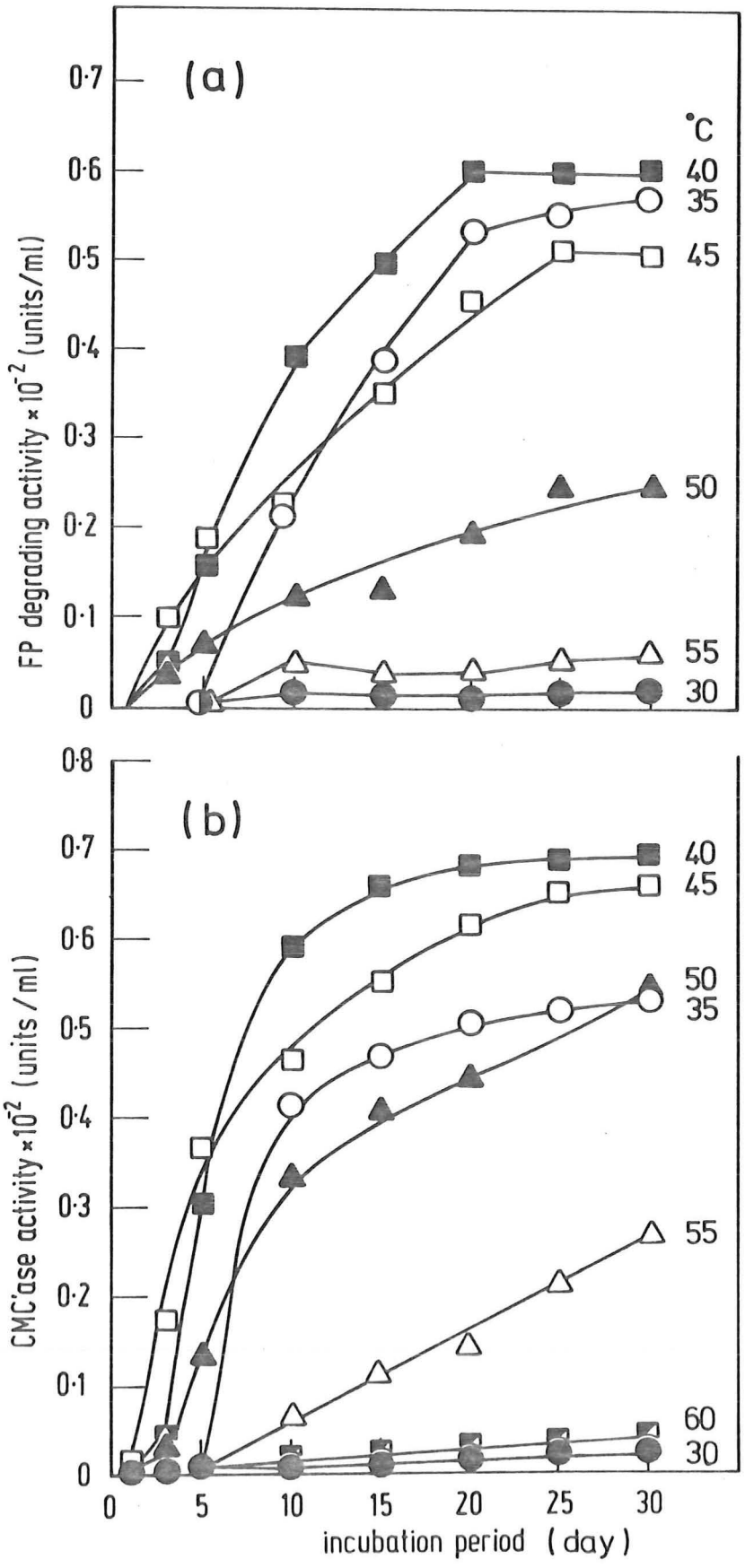
Effect of temperature on cellulase synthesis

Figure 8 demonstrates the effect of varying the growth temperature on cellulase production as measured by the ability of the culture filtrate to degrade filter paper and CMC.

The general pattern of enzymatic activity on both the substrates was similar at the temperatures tested. The optimum temperature for the production of the enzymic activities was 40°C and maximum activity was obtained after 20 days. Increasing the incubation temperature from 40 to 55°C resulted in a 90% decrease in the filter paper (FP) degrading activity and 60% of the carboxymethyl-cellulase (CMC'ase) activity. Incubation at 60°C eliminated almost all cellulase production. Thus, incubation at 40°C for 20 days was chosen for the production of cellulases in the following work. After 20 days, the integrity of the filter paper in the medium was lost completely and formed a thin slurry.

Figure 8. Influence of Temperature on Cellulase Production.

0.1 ml each of the cell-free culture filtrate was tested for activity on (a) filter paper and (b) CMC. Standard assays were used as described in Materials and Methods.


(a)
°C
40
35
45
50
55
30
FP degrading activity × 10⁻² (units/ml)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
(b)
40
45
50
35
55
60
30
CMC'ase activity × 10⁻² (units / ml)
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0
5
10
15
20
25
30
incubation period (day)

The pH of the culture medium changed during growth and these changes were similar for growth between 35 - 55°C. There was a drop within the first day from the initial pH of 6.5 to about 5.5, followed by a gradual rise to a final pH of 6.3 to 7.0. Main cellulase production occured at pH 6.3 - 6.8. The pH of the medium was 6.6 after growth at 40°C for 20 days.

General Properties of the Crude Enzymes from Culture Filtrate

Effect of temperature

Enzyme assays were carried out at pH 5.0 in the temperature range 30 - 80°C and the results are shown in Figure 9. Both β-glucosidase and CMC'ase activities showed temperature optimum at 70°C. At higher temperatures, the activities decreased sharply with 30% of the maximum activity remained at 80°C. The optimum temperature for FP degrading activity was lower. At 2 h reaction time, as opposed to the standard reaction time of 24 h, activity was greatest at 65°C compared with the optimum temperature of 60°C for 24 h reaction time. When the incubation temperature was lowered to 30°C, there was a 70 - 90% decrease of the maximum activity.

Effect of pH

Using a temperature of 70°C for β-glucosidase and CMC'ase and 60°C for FP degrading enzyme, the pH optima of the enzymes were determined and as shown in Figure 10.

An optimum pH of 5.0 was found for β-glucosidase and FP degrading enzyme and 4.3 for CMC'ase. If the reaction

Figure 9. Temperature Optimum for β-glucosidase and Cellulase Enzymes.

Enzyme activities were assayed in the standard systems at pH 5.0 except that the temperatures were varied as shown. Points shown are the average of duplicate determinations.

■ 24h reaction time

□ 2h reaction time

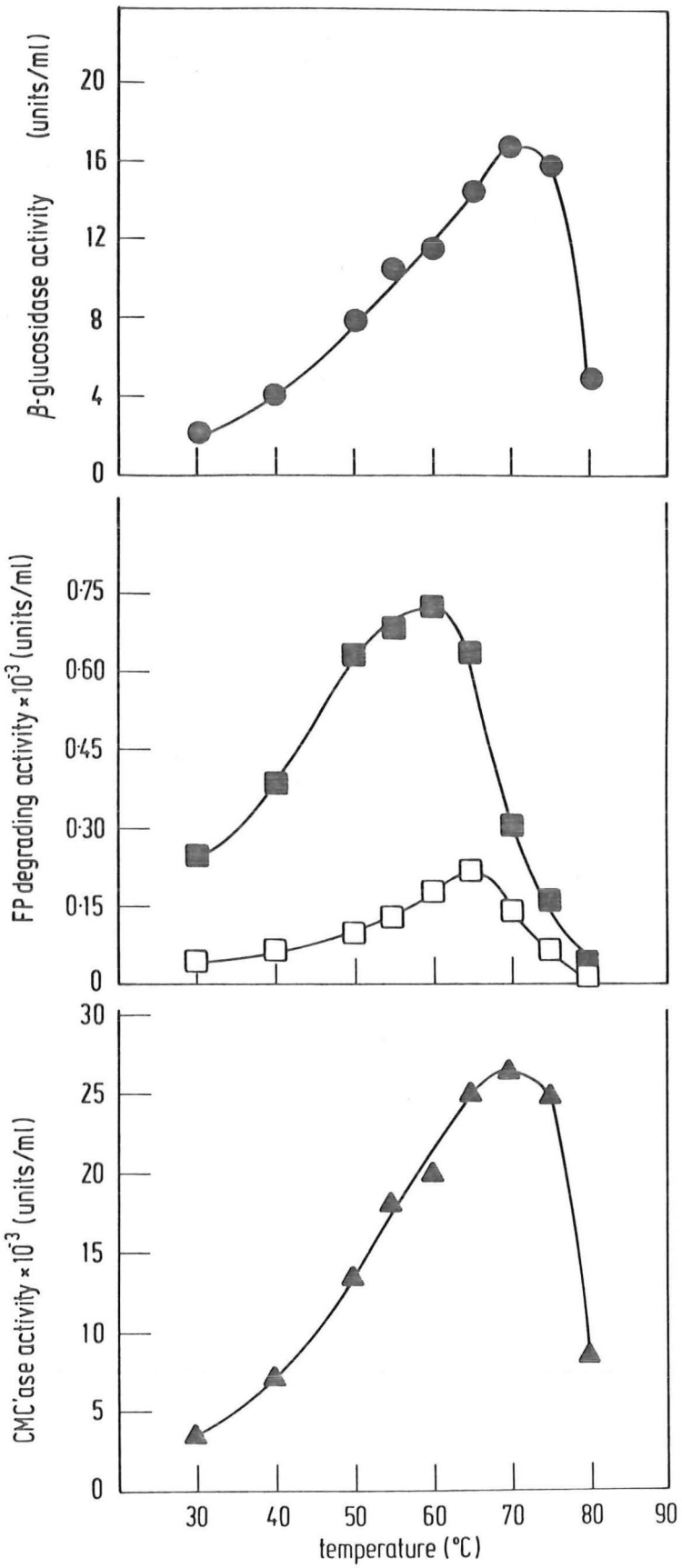
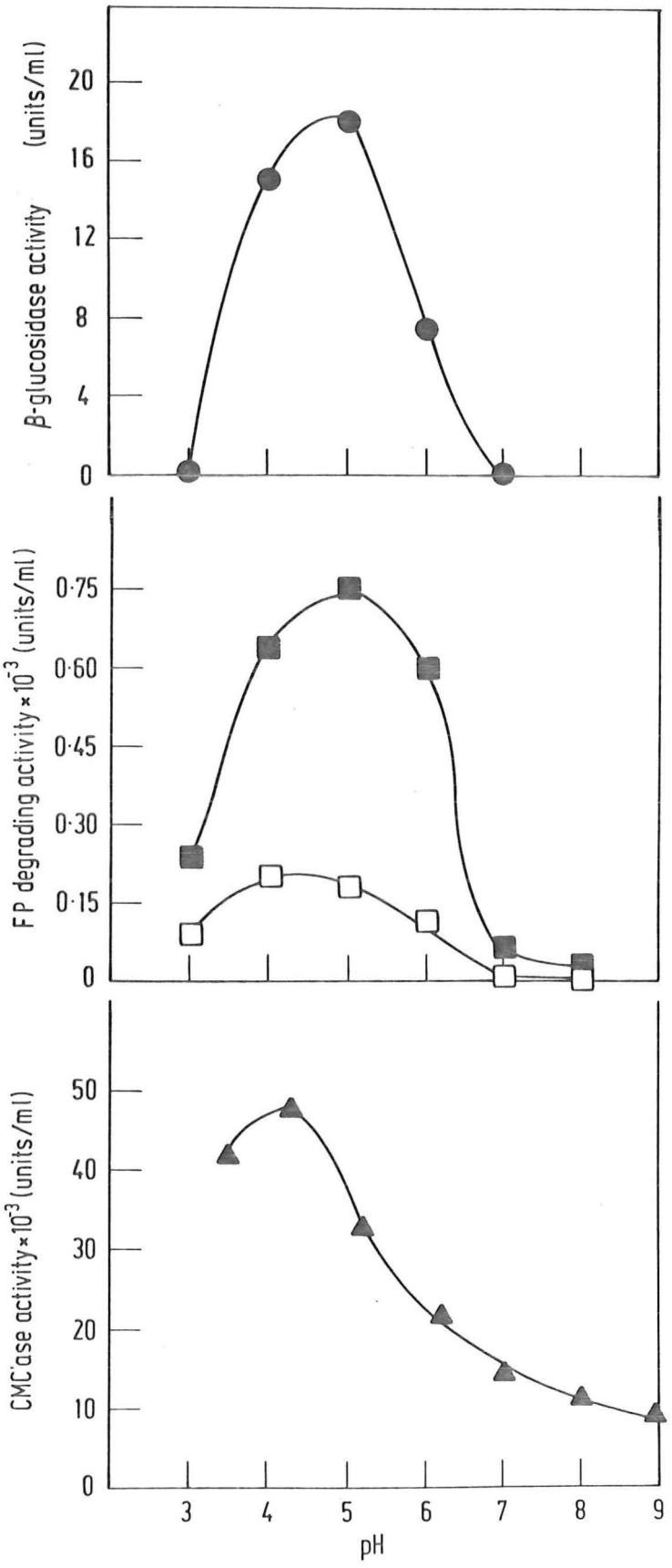

β-glucosidase activity (units/ml)
20
16
12
8
4
0
FP degrading activity × 10⁻³ (units/ml)
0·75
0·60
0·45
0·30
0·15
0
CMC'ase activity × 10⁻³ (units/ml)
30
25
20
15
10
5
0
30
40
50
60
70
80
90
temperature (°C)

Figure 10. Effect of pH on β-glucosidase and Cellulase Activities

The effect of pH on the enzymic activities were measured in the standard assay systems except that the buffer and pH were varied. Buffers used were citrate-phosphate (0.1M citric acid; 0.2M di-basic sodium phosphate) buffer (pH 3.0 - 7.0) and 0.2M Tris-HCl (pH 8.0, 9.0). Points were the average of two determinations.

■ 24 h reacting time

□ 2 h reacting time


β-glucosidase activity (units/ml)
20
16
12
8
4
0
FP degrading activity × 10⁻³ (units/ml)
0·75
0·60
0·45
0·30
0·15
0
CMCase activity × 10⁻³ (units/ml)
50
40
30
20
10
0
3
4
5
6
7
8
9
pH

time of the FP degrading activity was increased from 2 to 24 h, there was a shift in the pH optimum from 4.3 to 5.0. The cellulases exhibited a slightly broader pH range of activity than β-glucosidase.

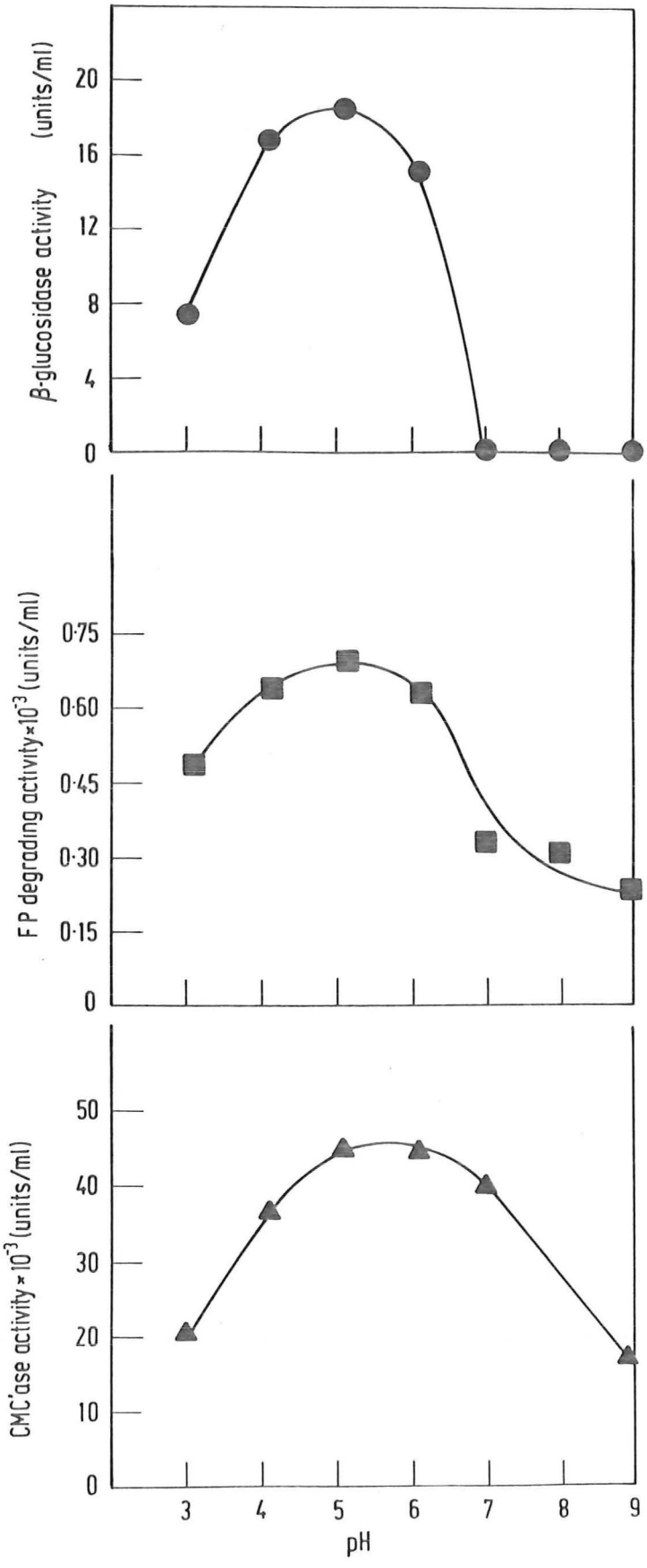
Effect of pH changes on stability

Figure 11 illustrates the effect of variations in pH on the stability of β-glucosidase and cellulases. The pH-stability curve for β-glucosidase resembles its pH-activity curve (Figure 10). The enzyme was stable over a pH range of 4.0 - 6.0. Cellulases exhibited a wider pH range of stability. Thirty percent of the pH 5.0 activity of FP degrading activity and 37% of the maximum CMC'ase activity still remained at pH 9.0.

Exploration of Procedures Designed for the Purification of β-glucosidase and Cellulases from *T. aurantiacus*

In an enzyme purification scheme it is desirable to reduce the amount of extraneous protein and concentrate the required activity by one or two of several standard procedures such as ammonium sulphate precipitation prior to using the more selective techniques such as ion-exchange chromatography or gel-filtration. However, in the purification of cellulases, this basic premise may not be sound because of the presence of several cellulolytic activities that are manifested under a single assay system. Hence the usefulness of a particular purification step, in isolating a single cellulolytic activity, may not be immediately apparent. The following procedures were tested for use as

Figure 11. Effect of pH on Stability of Crude Enzymes. Cell-free culture filtrates (0.2 ml) were mixed with the appropriate buffers (1.8 ml) and the resultant pH measured before incubation at 30°C for 3h. Same buffers were used as described for Fig. 10. 0.5 ml was withdrawn and assayed for enzyme activities in the standard assay systems except that the substrates were prepared in double-strength assay buffers. β-glucosidase and CMC'ase was assayed at 70°C in citrate-phosphate (0.2M citric acid; 0.4M di-basic sodium phosphate) buffer, pH 5.0 and 4.3, respectively, and FP degrading activity at 60°C in the same buffer just mentioned, pH 5.0. The pH of each incubation was checked after mixing the component solutions. Points were the average of two determinations.


β-glucosidase activity (units/ml)
20
16
12
8
4
0
FP degrading activity×10⁻³ (units/ml)
0·75
0·60
0·45
0·30
0·15
0
CMC'ase activity × 10⁻³ (units/ml)
50
40
30
20
10
0
3
4
5
6
7
8
9
pH

purification steps: ammonium sulphate precipitation; desalting on Bio-Gel P-2; Sephadex G-75 and Sephadex G-100 filtrations; alkaline swollen cellulose affinity column; DEAE-Sephadex A50 ion-exchange chromatography; iso-electric focusing and preparative disc-gel electrophoresis.

A. Ammonium sulphate precipitation

The initial step in the purification of cellulases from several microorganisms has been ammonium sulphate fractionation.

The 15-fold concentrated culture filtrate obtained from *T. aurantiacus* was fractionated at intervals of 10% ammonium sulphate saturation. It was found that all of the β-glucosidase and cellulase activities were confined to the 10 - 60% saturated fractions as shown in Table 2. This resulted in a 95% recovery of activities whilst removing 61% of the protein. Disc-gel electrophoresis of the various fractions was carried out and Figure 12 shows the number of protein bands associated with each fraction. No protein band was observed in the 10 - 20 and 20 - 30% saturated fractions. Increasing the sample load, 4 faint bands were observed in the 20 - 30% fraction (Figure 20); the positions of which corresponded with those shown in the 30 - 40% saturated fraction. In the 40 - 50% fraction only 2 bands were seen and 1 broad band in the 50 - 60% fraction.

B. Desalting on Bio-Gel P-2

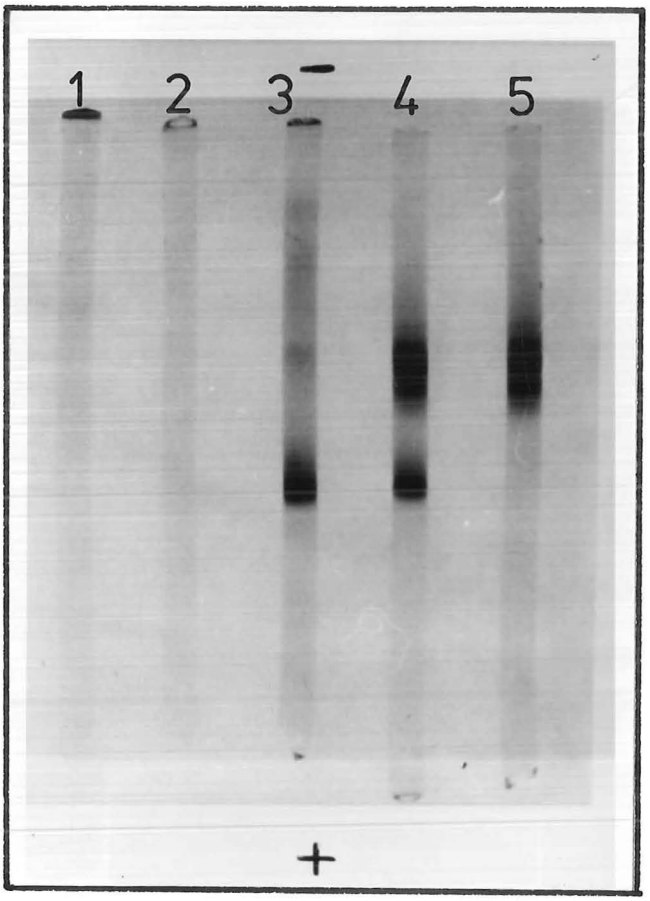
A number of reports have shown that cellulases have a strong affinity for cellulosic materials (Berghem *et al.*, 1976;

TABLE 2 Ammonium sulphate fractionation

% $(NH_4)_2SO_4$ saturation	Total protein (mg)	Total activity (units)		
		FP degrading activity x 10^3	CMC'ase activity x 10^3	β-glucosidase
10 - 20	36	0	2.75	66.6
20 - 30	51	5.50	5.75	266.6
30 - 40	86	15.75	200.00	300.0
40 - 50	120	37.80	136.00	31.6
50 - 60	76	10.00	2.75	8.2
60 - 100	189	0	0	0

Figure 12. Polyacrylamide Gel Electrophoresis.

The procedures employed were as described in Materials and Methods. Direction of migration was from top to bottom. Electrode terminals are as shown. Contents of gel tracks from left to right are protein from 1. 10-20%, 2. 20-30%, 3. 30-40%, 4. 40-50% and 5. 50-60% ammonium sulphate saturation fractions. Protein loads were 0.18, 0.25, 0.43, 0.60 and 0.38 mg. respectively.


1
2
3
4
5
−
+

Beguin & Eisen, 1977 and Binder & Ghose, 1978). In a pilot experiment, it was found that dialysis of the cellulase enzyme solution using cellulose acetate tubing resulted in a 25% loss of the cellulase activities, probably due to adsorption onto the surface of the tubing. In some cases, the dialysis tubing disintegrated, even at 4°C. Similarly, filtration through filter paper (Whatman No. 1) also resulted in 20% loss of the enzyme activities. Thus, the use of cellulose acetate tubing for dialysis and filtration through filter paper was avoided. In this work, gel filtration on Bio-Gel P-2, a biologically inert, crosslinked synthetic methylene-bis-acrylamide polymer, was adopted for desalting purposes.

The concentrated dark brown enzyme solutions from the ammonium sulphate precipitation steps were desalted on a column of Bio-Gel P-2. The elution profile (Figure 13) shows that all of the enzymic activities were eluted in the void volume. In addition some of the pinkish brown pigment of low molecular weight was also removed as indicated by the two smaller peaks.

C. Gel filtration on Sephadex G-75

Wood (1968) separated a low molecular weight CMC'ase component of a concentrated cell free filtrates from *T. koningii* by chromatography on Sephadex G-75.

A portion of the desalted enzyme solution (10 - 40% ammonium sulphate fraction) was fractionated on a Sephadex G-75 column. Figure 14 demonstrates that the β-glucosidase was partially separated from the cellulase peak. The FP

Figure 13. Desalting on Bio-Gel P-2.

Conditions were as described in Results.

absorbance at 280 nm.

β-glucosidase activity.

FP degrading activity.

CMC'ase activity.

Fractions under the bar were pooled for further purification.

10⁻³ x FP degrading activity (units / ml)
10⁻³ x CMCase activity (units / ml)
β-glucosidase activity (units / ml)
absorbance at 280 nm
fraction no.
0
8
16
24
32
40
48
56

Figure 14. Gel Filtration on Sephadex G-75.

2.0 ml (30 mg) of the enzyme solution (20-40% ammonium sulphate fraction) obtained after dialysis through Bio-Gel P-2 was applied to the Sephadex G-75 column (1.5 x 25 cm) equilibrated with 0.05 M ammonium formate buffer pH 5.0. Fractions of 2.5 ml were collected and assayed for β-glucosidase and cellulase activities.

β-glucosidase activity.

FP degrading activity.

CMC'ase activity.

10⁻¹ x FP degrading activity (units / ml)
10⁻¹ x CMCase activity (units / ml)
β - glucosidase activity (units / ml)
fraction no.
0
1
2
3
4
5
6
7
8
9
10
3
6
9
12
15
18
21
24
27
30
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6

degrading activity was almost coincident with the CMC'ase activity. Thus, an attempt to further separate the β-glucosidase from cellulases by chromatography on Sephadex G-100 seemed promising.

D. Gel filtration on Sephadex G-100

Pettersson (1963) revealed the presence of at least four components active on CMC and *p*-nitrophenyl-β-D-glucoside, in a commercial preparation of *A. niger* cellulase, after gel filtration on Sephadex G-100.

Sephadex G-100 was successful in separating β-glucosidase activity from the cellulolytic activities using solutions partially purified by ammonium sulphate precipitation and Bio-Gel P-2. The elution profile obtained is shown in Figure 15. Two protein peaks were evident and most of the β-glucosidase was eluted in the first peak. Only trace amounts of the cellulolytic activities were associated with the β-glucosidase component. The second peak contained most of the cellulase activities. Repeated chromatography on Sephadex G-100 of the active fractions pooled as shown resulted in complete separation of the β-glucosidase from the cellulase activities (not shown).

E. Affinity chromatography on alkaline-swollen cellulose

Alkaline-swollen cellulose was introduced by Hash & King (1958) for the purification of cellulases from *M. verrucaria* and subsequently used by Li & King (1963) in isolating eight partially purified components from a commercial crude *A. niger* cellulase. In later years this

Figure 15. Gel Filtration on Sephadex G-100.

Details of the conditions are given in the text (pg 90).

Absorbance at 280 nm.

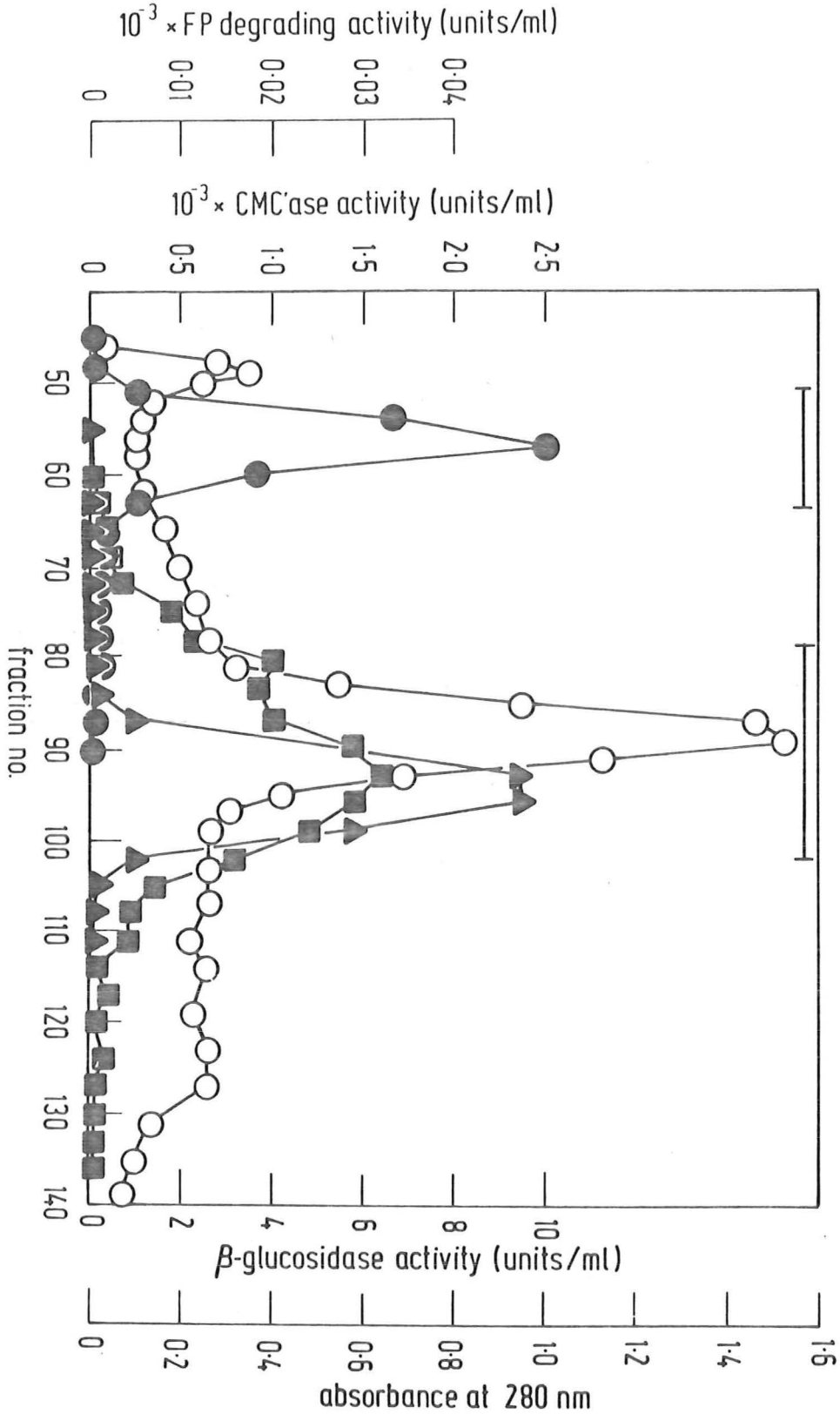
β-glucosidase activity.

FP degrading activity.

CMC'ase activity.

Fractions under the bars were pooled.

3.5 ml of the 10-40% ammonium sulphate fraction was applied to the Sephadex G-100 column.


10⁻³ × FP degrading activity (units/ml)
0
0·01
0·02
0·03
0·04
10⁻³ × CMC'ase activity (units/ml)
0
0·5
1·0
1·5
2·0
2·5
fraction no.
50
60
70
80
90
100
110
120
130
140
0
2
4
6
8
10
β-glucosidase activity (units/ml)
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
absorbance at 280 nm

technique of affinity chromatography has been expanded by the use of Avicel (Li *et al.*, 1965) and cotton gauze (Okada *et al.*, 1968) as column adsorbents.

A typical distribution of protein and enzyme activities after fractionation on alkaline-swollen cellulose column of the partially purified cellulase component obtained from repeated chromatography on Sephadex G-100 is shown in Figure 16. The protein peak that was excluded from the column in the void volume was rich in FP degrading activity. This activity continued to be eluted from the column up to a hundred fractions and the fraction collection stopped. The CMC'ase was slightly retarded on an alkaline-swollen cellulose column and excluded from the column as a broad peak. Fractions collected were also assayed for reducing sugars formed as a result of degradation of the cellulose by cellulolytic enzymes.

F. Ion-exchange chromatography on DEAE-Sephadex A-50

Chromatography on DEAE-Sephadex has been widely used in the purification of cellulases from a variety of micro-organisms. Wood (1968) was able to separate a cellulase component (C_1) from the CMC'ase (C_x) and β-glucosidase activities by chromatography on DEAE-Sephadex from a cell-free culture filtrate of *T. koningii*. Preliminary experiments on DEAE-Sephadex with the desalted crude enzyme extract using salt or pH gradient elution had failed to remove the enzymes from the column once the proteins had been bound to the ion-exchanger. This problem was not encountered with batch separation. The elution profile (Figure 17) shows that all

Figure 16. Alkaline-Swollen Cellulose Chromatography.

Cellulase enzymes (2.0 ml), partially purified by ammonium sulphate precipitation and re-cycling twice on a Sephadex G-100 column, were used. A sample containing 20 mg protein in 0.05M ammonium formate buffer pH 5.0 was applied to a column (2.7 x 30 cm) of alkaline-swollen cellulose. Elution with the same buffer was at a rate of 18 ml/h. Fractions of 2.5 ml were collected.

Absorbance at 280 nm.

Reducing sugar.

FP degrading activity.

CMC'ase activity.

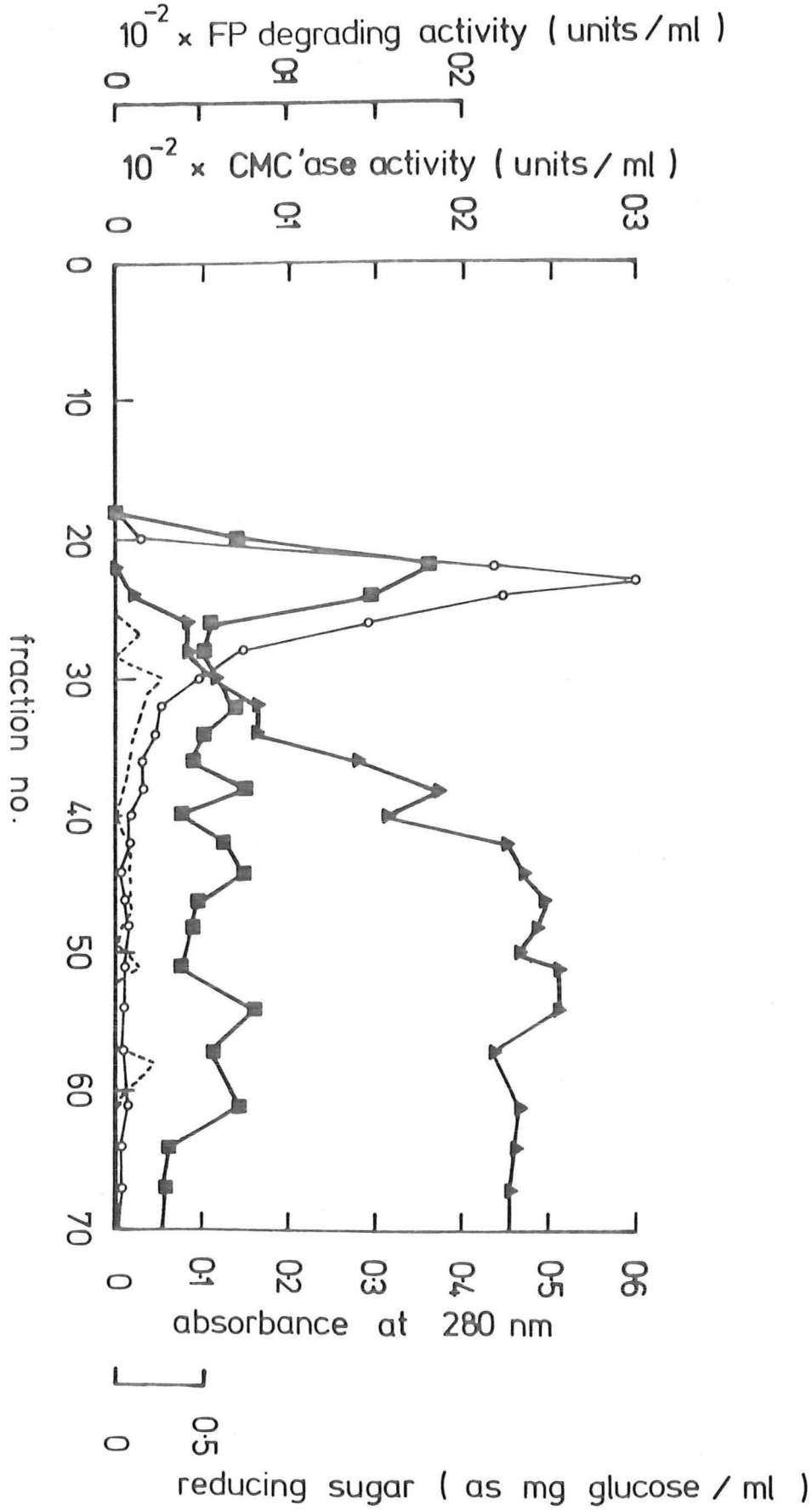

10^{-2} x FP degrading activity (units / ml)
0
0·1
0·2
10^{-2} x CMC'ase activity (units / ml)
0
0·1
0·2
0·3
fraction no.
0
10
20
30
40
50
60
70
0
0·1
0·2
0·3
0·4
0·5
0·6
absorbance at 280 nm
0
0·5
reducing sugar (as mg glucose / ml)

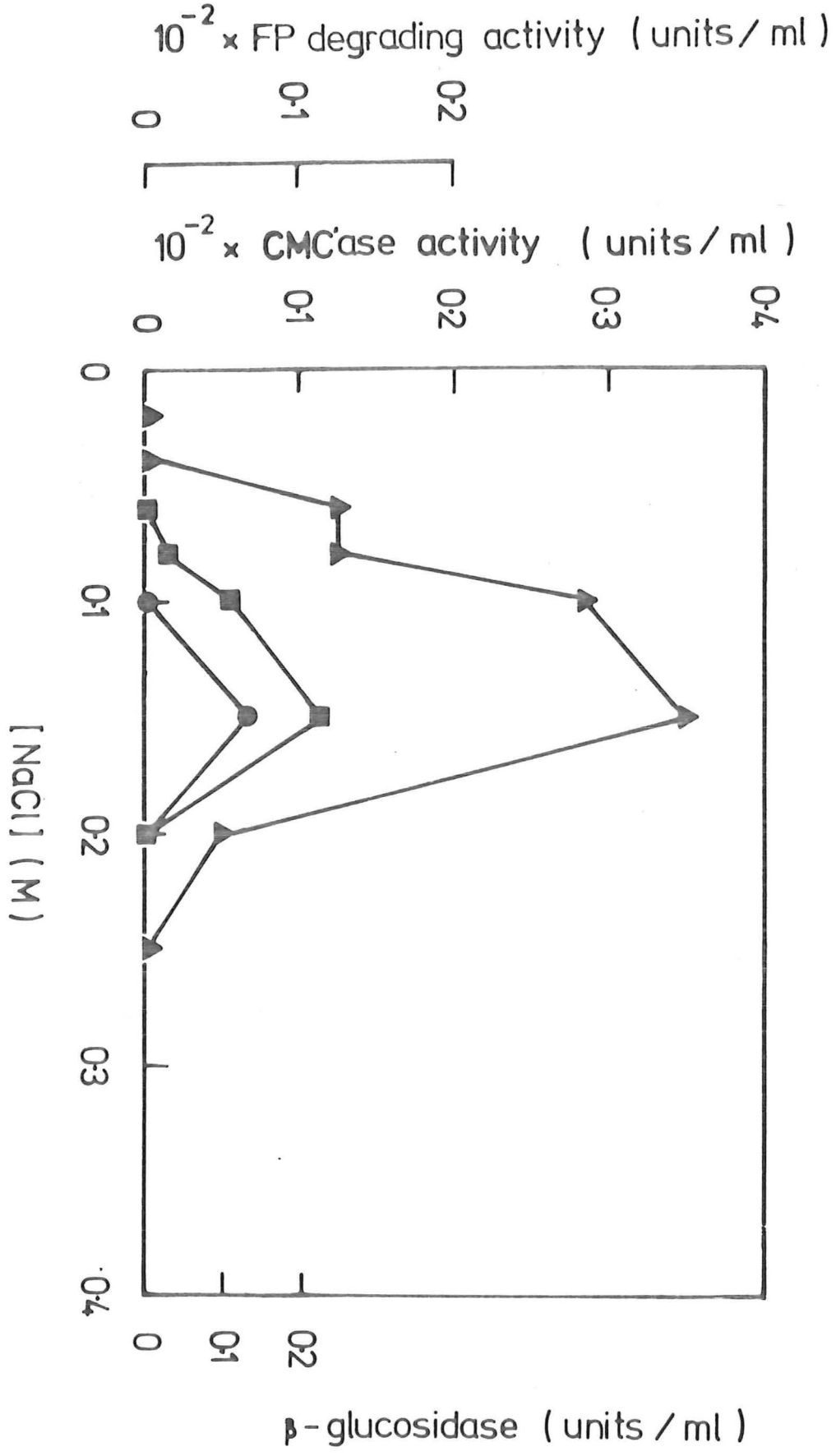
Figure 17. DEAE - Sephadex A-50 Chromatography.

0.5 ml dialysed enzyme solution (10-40% ammonium sulphate fraction) containing 7.5 mg protein was stirred in a beaker containing 0.5 g of the ion-exchanger equilibrated with 15 ml of 0.2M Tris-NaOH buffer, pH 6.0. The mixtures were equilibrated for an hour before filtering through a sintered glass funnel (G2) and the residue was washed with the buffer solution. The proteins on the residual ion-exchanger were eluted fractionally by resuspending the slurry in buffer solutions of higher ionic strength as shown. Fractions (15 ml) collected were assayed for the activities.

β-glucosidase activity.

FP degrading activity.

CMC'ase activity.


10^{-2} x FP degrading activity (units / ml)
0
0·1
0·2
10^{-2} x CMC'ase activity (units / ml)
0
0·1
0·2
0·3
0·4
[NaCl] (M)
0
0·1
0·2
0·3
0·4
β - glucosidase (units / ml)

the activities were eluted as a single peak when the solution was approximately 0.2 M with respect to sodium chloride. Attempts to separate the various components by changes in pH of the eluting solution after the proteins were adsorbed onto the ion-exchanger at pH 6.0 (0.1 M citric acid; 0.2 M di-basic sodium phosphate) yielded similar results (not shown). All the proteins were eluted as a single peak when the pH of the solution had dropped to 2.5. It was subsequently found that β-glucosidase and cellulases had close iso-electric points.

G. Isoelectric focusing

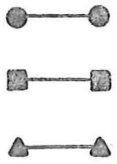
Analytical isoelectric focusing of the enzyme solution from Bio-Gel P-2 was carried out in polyacrylamide gels as described in Materials and Methods. The results indicated that the preparation was isoelectric at acidic pH values. Preparative isoelectric focusing, therefore, was performed using ampholine pH 4 - 6. The elution profile (Figure 18) showed that the enzymes were focused very close together at pH values below 4.0. Hence, isoelectric focusing under the conditions used did not appear to be a suitable method for the purification of the enzymes.

Final Purification Scheme in Detail

The final purification procedure is summarised in Figure 19. Apart from column chromatography which was carried out at room temperature, all operations were conducted at 0 - 4^{o}C. Concentration of the enzyme fractions was achieved by lyophilisation; in this procedure the salt concentration

Figure 18. Preparative Isoelectric Focusing.

Conditions were as described under Materials and Methods. About 33 mg protein obtained from Bio-Gel P-2 filtration was electro-focused.



β-glucosidase activity.

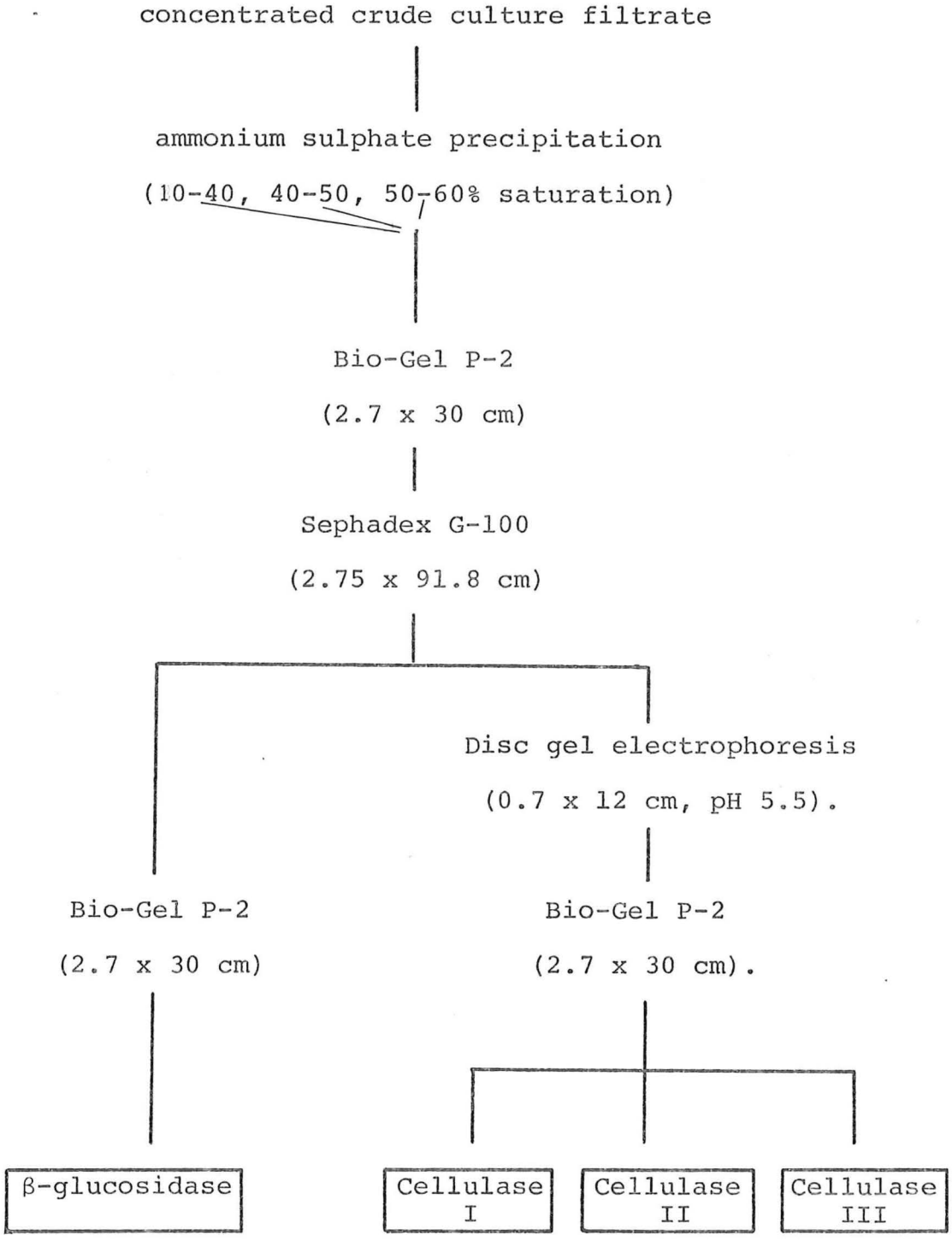
FP degrading activity.

CMC'ase activity.

pH measured at room temperature.

10⁻² × FP degrading activity (units / ml)
0
0·1
0·2
0·3
0·4
0·5
10⁻² × CMCase activity (units / ml)
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
pH 4·0
pH 2·2
0
10
20
30
40
50
60
70
fraction no.
0
0·1
0·2
0·3
0·4
0·5
β-glucosidase (units / ml)
0
2
4
6
8
10
pH

Figure 19. Fractionation Scheme for the Purification of β-glucosidase and Cellulolytic Enzymes from *T. aurantiacus*.


concentrated crude culture filtrate
ammonium sulphate precipitation
(10-40, 40-50, 50-60% saturation)
Bio-Gel P-2
(2.7 x 30 cm)
Sephadex G-100
(2.75 x 91.8 cm)
Disc gel electrophoresis
(0.7 x 12 cm, pH 5.5).
Bio-Gel P-2
(2.7 x 30 cm)
Bio-Gel P-2
(2.7 x 30 cm).
β-glucosidase
Cellulase I
Cellulase II
Cellulase III

did not increase because of the volatile nature of the buffer.

Solid ammonium sulphate was slowly added to 100 ml of stirred crude culture filtrate to give 10 - 40, 40 - 50 and 50 - 60% saturated fractions. The solution was stirred for a further 30 min. after addition of ammonium sulphate. The solution was centrifuged (10,000 g for 30 min.) and the precipitate resuspended in 20 ml of 0.05 M ammonium formate buffer, pH 5.0. Insoluble material was removed and discarded by centrifugation (10,000 g for 30 min.). Fractions containing the various ammonium sulphate concentrations were treated separately using the following purification steps.

Fractions from the ammonium sulphate precipitation were desalted on a Bio-Gel P-2 (100 - 200 mesh) column (2.7 x 30 cm) equilibrated with the ammonium formate buffer (flow rate approx. 100 ml/h) with a void volume of 50 ml. Fractions of 5 ml were collected. The eluate (approx. 60 ml) was lyophilised and the residue resuspended in 5 ml of the formate buffer. Any undissolved material was removed and discarded by centrifugation (10,000 g for 30 min.).

The desalted samples were layered onto a Sephadex G-100 (particle size = 40 - 120 μm) column (2.75 x 91.8 cm) equilibrated with ammonium formate buffer (flow rate approx. 45 ml/h). Fractions of 3.5 ml were collected and those fractions with more than 30% of the activity of the peak fraction were pooled as shown in Figure 15 and lyophilised. The same formate buffer (5.0 ml) was used to resuspend the residue. The suspension was centrifuged (10,000 g for 30 min.) to remove undissolved material. Recycling through the same column of Sephadex G-100 was necessary in order to completely separate the β-glucosidase from the cellulases.

The purified β-glucosidase was finally desalted on a Bio-Gel P-2 column and the cellulases purified by disc-gel electrophoresis. Figure 20 shows the purified β-glucosidase removed from the cellulases after chromatography on Sephadex G-100.

In the purification of cellulases by disc-gel electrophoresis fractions containing 10 - 30 and 30 - 40% ammonium sulphate saturation were combined. 0.1 ml of the partially purified cellulase enzyme solution was mixed with 40 µl of 80.7% glycerol and 10 µl bromophenol blue before layering on top of each gel tube. Electrophoresis was carried out as described under Materials and Methods. Unstained gels were scanned at 280 nm on a Joyce Loebl u.v. scanner (Figure 21) and sliced. Based on the position of the protein bands in each gel, the sliced gels of the same position were combined. Protein was eluted in formate buffer, pH 5.0, by grinding the gels in uniform PTFE pestle/glass body homogenisers with repeated washings. The gelatinous material was removed by centrifuging at 10,000 g for 30 min. The supernatant obtained was filtered through a millipore filter (0.45 µm pore size) and the filtrate concentrated by lyophilisation. The dried material was dissolved in 3.0 ml of formate buffer and desalted on a Bio-Gel P-2 column. The active fractions were pooled and the resulting purified cellulases were used for subsequent studied.

The purification scheme for cellulase III is given in Table 3.

Figure 20. Polyacrylamide Gel Electrophoresis.

Conditions for polyacrylamide gel electrophoresis were as described under Materials and Methods. Direction of migration was from top to bottom. Electrode terminals as shown.

1. Purified β-glucosidase from 2. the 10-30% saturated fraction; 3. purified β-glucosidase from 4. the 30-40% saturated fraction; 5. 40-50% and 6. 50-60% ammonium sulphate saturated fraction. Protein loads were 0.05, 0.48, 0.06, 0.85, 1.88 and 1.15 mg, respectively.

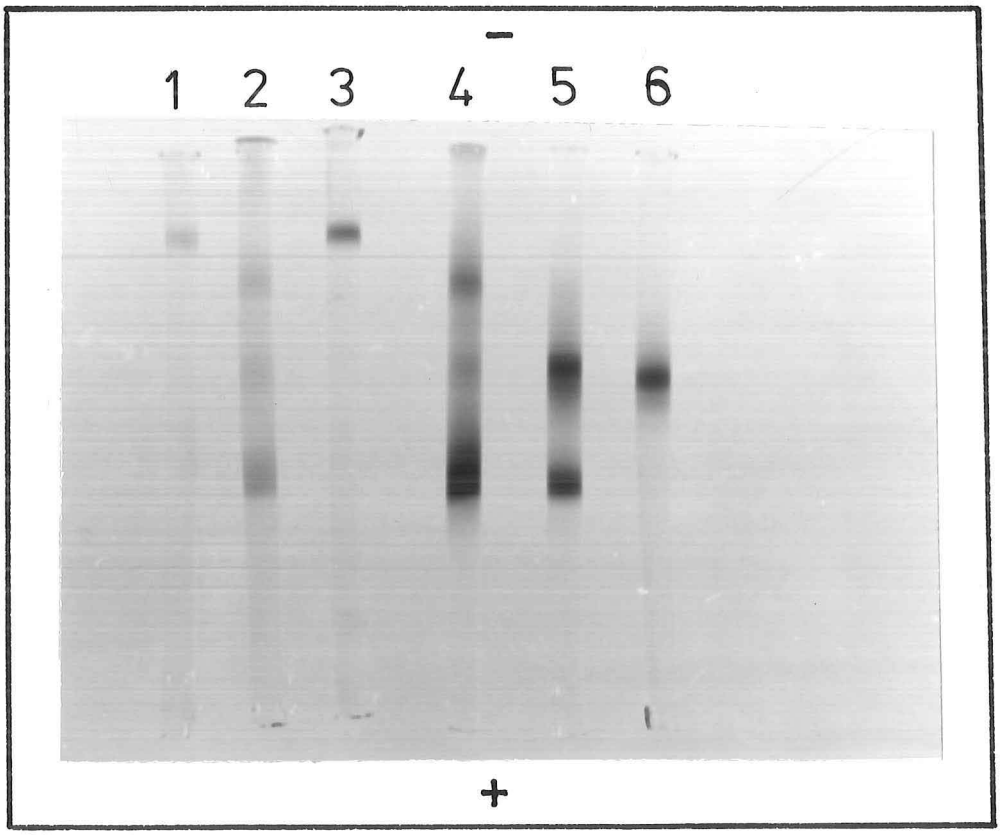

−
1 2 3 4 5 6
+

Figure 21. Densitometric Tracing of Dis-Gel Electrophoresis.

Unstained gels were scanned under U.V. light on a Joyce Loebl U.V. scanner. Direction of migration was left to right. (a) 10-40%, (b) 40-50% (c) 50-60% ammonium sulphate saturated fractions after chromatography on Sephadex G-100.

CI was isolated after gel electrophoresis of the 10-40% ammonium sulphate fraction.

CII was isolated from the gels of the 40-50 and the 50-60% ammonium sulphate fractions.

CIII was isolated from the gels of the 10-40% and the 40-50% ammonium sulphate fractions.

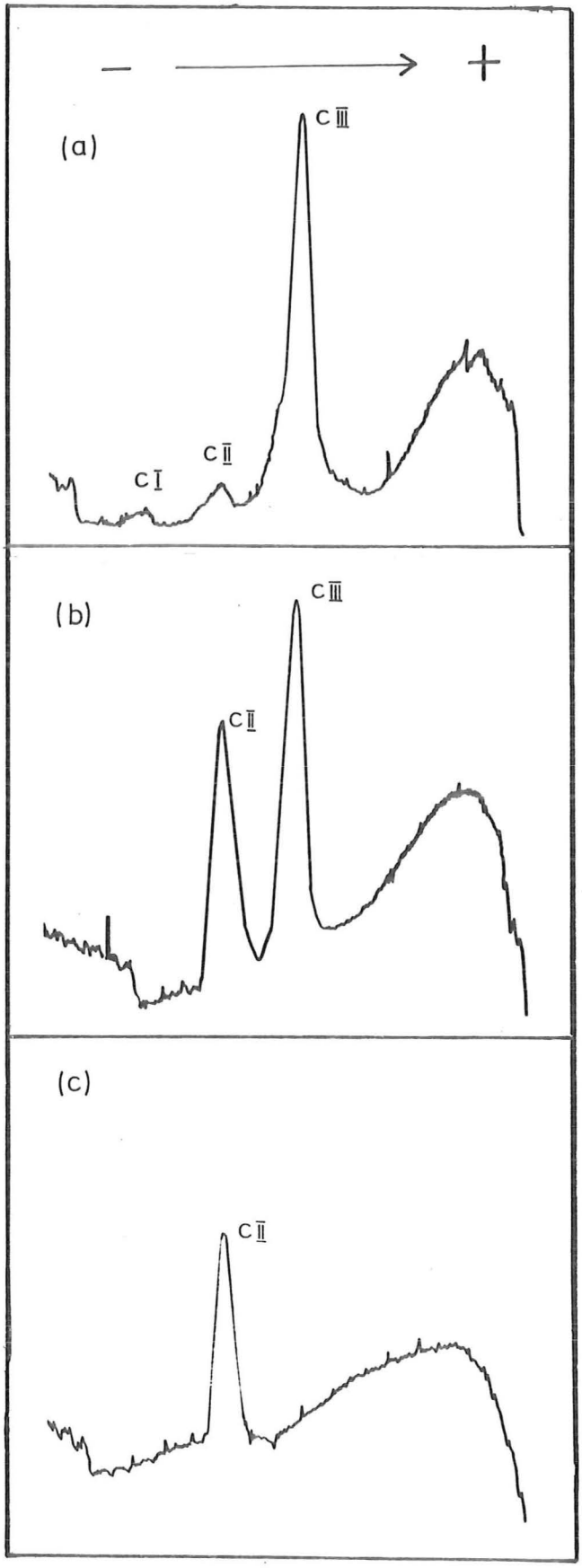

(a)
C Ⅲ
C Ⅱ
C Ⅰ
(b)
C Ⅲ
C Ⅱ
(c)
C Ⅱ

TABLE 3 Purification of a cellulase (Cellulase III) from *T. aurantiacus*

Fraction	Volume (ml)	Total Activity x 10^3 (units)	Total Protein (mg)	Specific Activity x 10^3 (units/mg)	Purification
Crude extract	1510.0	195.0	1087.2	0.17	-
30 - 40% saturated $(NH_4)_2SO_4$ ppt.	20.0	160.0	86.0	1.86	10.94
Bio-Gel P-2 eluate	57.3	148.5	67.0	2.21	13.0
Sephadex G-100 eluate (2nd Chromatography)	130.0	104.0	41.6	2.50	14.7
Disc Gel Electrophoresis eluate	20.0	150.0	23.6	11.02	64.8

Enzyme Purity

To examine the homogeneity of the purified enzyme preparations, analytical polyacrylamide disc-gel electrophoresis was carried out in citrate-phosphate buffer (0.1 M citric acid; 0.2 M di-basic sodium phosphate), pH 5.5, according to the technique introduced by Ornstein and Davis (1964) as described in Materials and Methods. The purified enzymes migrated as a single band as shown in Figure 22, indicating that the enzymes are electrophoretically homogenous. Scanning of the unstained gels on a Joyce Joebl u.v. scanner also indicated homogeneity of the enzyme preparations.

The first protein band (in descending order) in the gel was found to be a β-glucosidase active against *p*-nitrophenyl-β-D-glucoside and cellobiose. The other three purified enzymes possessed cellulolytic activity on native cellulose and/or modified soluble cellulose. No attempt was made at this stage to identify those cellulase enzymes with the "C_1" and "C_x" enzymes postulated by Reese and his co-workers (1950). For convenience, the cellulases are referred to as Cellulase I, Cellulase II and Cellulase III respectively in the following work, according to the positions they occupied in the gel in descending sequence.

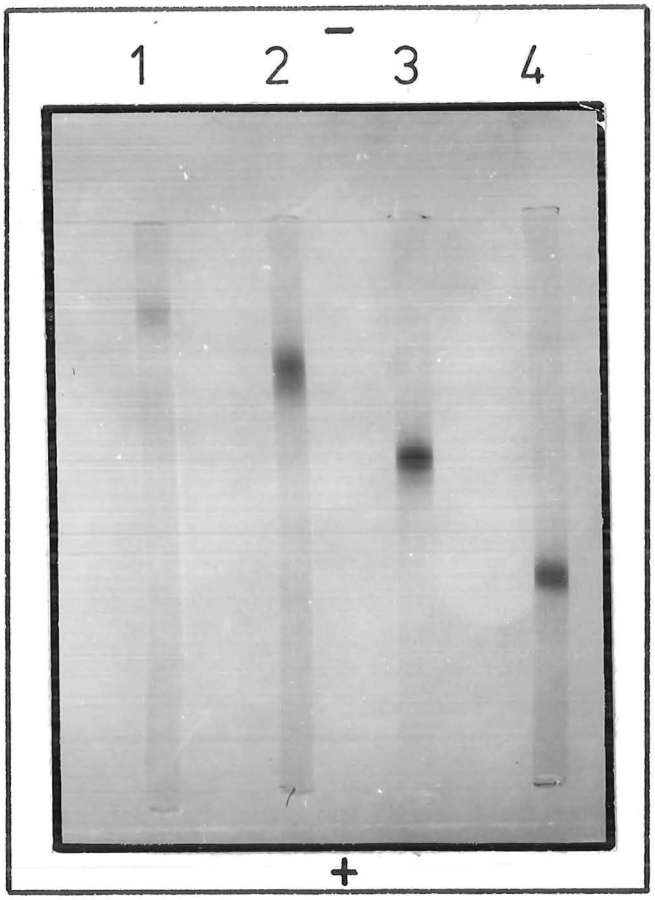
Enzyme Stability

The purified β-glucosidase and cellulases were stable for at least a year when stored as frozen solutions at -20°C. In addition, dilute solutions (5 μg/ml) could be kept at 4°C for several days without significant loss of activities.

Figure 22. Polyacrylamide Gel Electrophoresis of the Purified Enzymes.

0.1 ml of the purified enzyme solutions were subjected to electrophoresis in 7.5% polyacrylamide gel for 23 h at 7.0 mA/tube (0.7 x 12.0 cm). Other conditions including staining of the gels were as described in Materials and Methods. Protein loads were 1. 21.0; 2. 48.0; 3. 25.0; 4. 20.4 µg, respectively. Migration was from top to bottom. Electrode terminals as shown.

1. β-glucosidase
2. Cellulase I
3. Cellulase II
4. Cellulase III


1
2
3
4
−
+

Molecular Weight Determination

The molecular weights of the purified enzymes were determined by gel filtration through a calibrated column of Bio-Gel P-60. The enzymes were chromatographed as single peaks under these conditions. A linear relationship was obtained by plotting the elution volumes of the standard proteins against log molecular weights (Figure 23). The molecular weight of the enzymes determined from this curve were estimated to be 85,000 for β-glucosidase, 78,000 for cellulase I, 48,000 for cellulase II and 34,000 for cellulase III.

The molecular weight of the enzymes were also determined by SDS-electrophoresis. Typical protein band patterns after electrophoresis and staining as described in Materials and Methods are shown in Figure 24. All except cellulase I showed one major band with one or more faint bands associated with it. In the case of cellulase I, several faint bands were observed. Some of these bands were also evident in gels containing the standard proteins. Figure 25 shows the plot of the logarithms of the molecular weights against the electrophoretic mobilities. The relative mobility of the major band of β-glucosidase, cellulase II and III indicated their molecular weights to be about 98,000, 51,000 and 34,500 respectively. The faint bands of cellulase I had molecular weights ranging from 25,500 to 80,000.

Isoelectric Point Determination

Preparative isoelectric focusing revealed that the enzymes were focused at pH values below 4.0. For this reason,

Figure 23. Estimation of the Molecular Weights of β-glucosidase and Cellulases on a Calibrated Column of Bio-Gel P-60.

Conditions were described in Materials and Methods. Standard proteins were 1, α-amylase; 2, α-chymotrysin; 3, bovine serum albumin and 4, lactoferrin. The open circles indicate the elution volumes of the β-glucosidase and cellulases. The molecular weights of marker proteins (except Lactoferrin) were from Smith (1968).

○ β-glucosidase

◇ Cellulase I

□ Cellulase II

△ Cellulase III

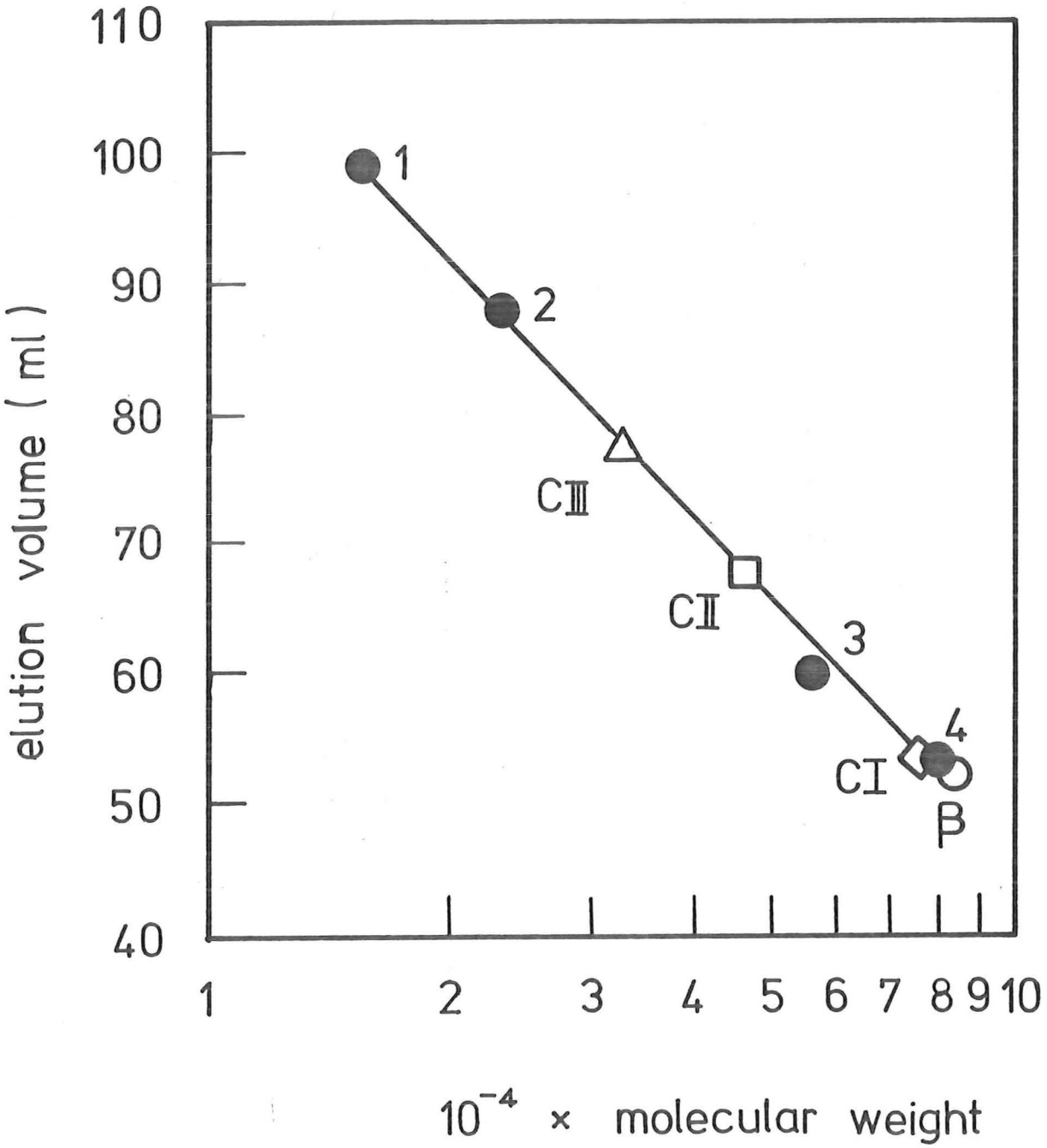

110
100
90
80
70
60
50
40
elution volume (ml)
1
2
CIII
CII
3
4
CI
β
1
2
3
4
5
6
7
8
9
10
10⁻⁴ × molecular weight

Figure 24. SDS Gel Electrophoresis.

Conditions were as described in Materials and Methods. Electrophoresis was from top to bottom. The gels shown contained, 1. bovine serum albumin, ovalbumin, chymotsypsinogen, myoglobin; 2, lactoferrin; 3. cytochrome c; 4. β-glucosidase; 5. Cellulase I; 6. Cellulase II, and 7. Cellulase III. Protein loads were 10 µg each for the standard proteins, 21.0 µg for β-glucosidase, 48.0 µg for Cellulase I, 25.0 µg for Cellulase II and 20.4 µg for Cellulase III.


−
1 2 3 4 5 6 7
+

Figure 25. Determination of Molecular Weights of Purified β-glucosidase and Cellulases by SDS gel electrophoresis.

The mobilities of the standard proteins relative to bromophenol blue were plotted against their molecular weights. Proteins used for standard curve were: 1, bovine serum albumin; 2, ovalbumin; 3, chymotrypsinogen; 4, myoglobin; 5, lactoferrin and 6, cytochrome C. The open circles indicate the relative mobility of the β-glucosidase and cellulases.

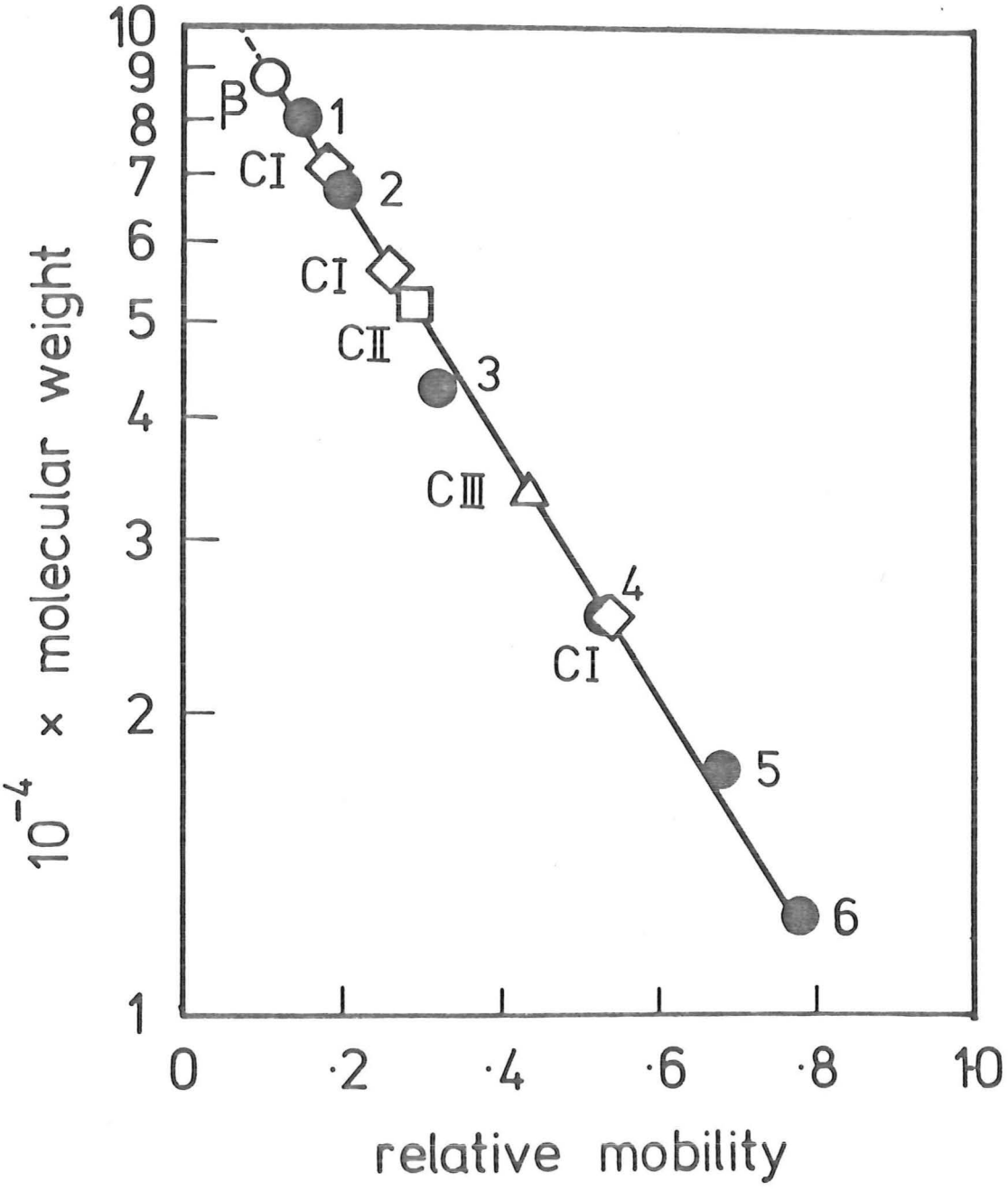
○ β-glucosidase

◇ Cellulase I

□ Cellulase II

△ Cellulase III

The molecular weights of the marker proteins except lactoferrin were from tables compiled by Smith (1968).


10
9
8
7
6
5
4
3
2
1
β
1
CI
2
CI
CII
3
CIII
4
CI
5
6
10^{-4} × molecular weight
0
·2
·4
·6
·8
10
relative mobility

a commercially available ampholyte solution covering a lower pH range of 3.5 to 5.0 was used in the determination of isoelectric points of the purified enzymes in polyacrylamide gels. Figure 26 shows typical protein band patterns after electrofocusing for 8 h. The isoelectric points of the enzymes were low with the exception of cellulase I which again showed up as a number of faint bands mostly covering the pH range of 3.8 to 5.0. Attempts to measure this property accurately using electrofocusing failed since the isoelectric points of most of the enzymes were close to pH 3.0 and therefore at the lower limit of the range of ampholytes available. However, from the results obtained here, the isoelectric points of β-glucosidase, cellulase II and III were estimated to be 2.4 - 2.5, 2.3 - 2.7 and 2.7 - 3.2 respectively. The enzymes showed a tendency to precipitate towards the end of the electrofocusing experiments. This was possibly because the enzyme concentrated very close to the anode, or because of the high concentration of the enzyme which built up at a pH where enzyme solubility was low, a common feature with enzymes at or near isoelectric points.

Carbohydrate Content

The carbohydrate contents of the purified enzyme preparations were determined by the method of Herbert, *et al.*, (1971), and are summarised in Table 4. Protein concentrations in the samples were estimated by the method of Lowry *et al.*, (1951).

Figure 26. Analytical Isoelectric Focusing of the Purified Enzymes.

Conditions were given in Materials and Methods. Electrode terminals as shown. Protein loads were 1. 35.0 µg for β-glucosidase; 2. 48.0 µg for Cellulase I; 3. 12.5 µg for Cellulase II and 4. 16.5 µg for Cellulase III.

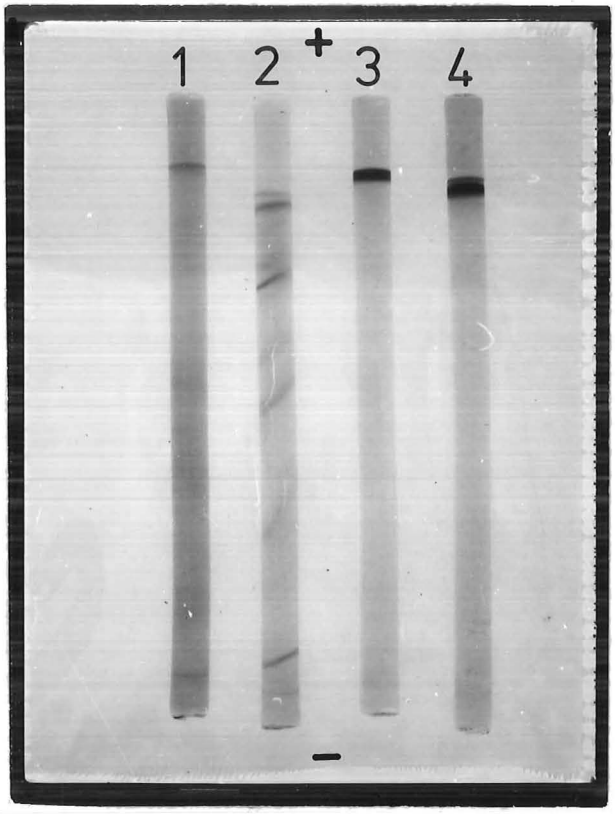

1
2
+
3
4
−

TABLE 4 Carbohydrate content in the enzyme preparations

Enzyme	Protein (µg)	Carbohydrate (µg as glucose)	% of Carbohydrate by weight
β-glucosidase	28	14	33.0
Cellulase I	12	0.7	5.5
Cellulase II	109	3.0	2.6
Cellulase III	136	2.5	1.8

0.5 ml of the enzyme solutions of appropriate dilution were used for the determination of protein and carbohydrate content. The values are the average of duplicate sample.

The types of carbohydrate and the types of linkages between carbohydrate and protein were not determined.

Substrate Specificity

To study the specificities of the purified β-glucosidase and cellulases, a number of different glycosidic compounds was tested as substrates, to obtain information regarding the glycosyl linkage and glycosyl group requirements of the enzymes. All incubations were carried out in citrate-phosphate buffer (0.1 M citric acid; 0.2 M di-basic sodium phosphate) pH 5.0 except CMC which was performed at pH 4.5. The results are shown in Table 5. Comparison of the relative ease with which the various substrates were degraded by each enzyme was difficult because of the varying incubation periods and enzyme concentrations used. Despite these limitations, it was noted that some compounds were more readily hydrolysed than others. Comparison of the various enzymic activities on each substrate was made possible by using equal amounts of each enzyme in the reaction mixtures and incubating under similar conditions for

TABLE 5 Substrate specificity of β-glucosidase and cellulases from *T. aurantiacus*

Substrate	Substrate conc. (mg/ml)	Linkage type(s)	Incubation time (h)	Final enzyme conc. (μg/ml)	Reducing sugar as glucose equivalent (μg)			
					β-glucosidase	Cellulase I	Cellulase II	Cellulase III
Cotton yarn	20	β-1,4	24	4.1	0	3	7	0
Solka-Floc	20	β-1,4	24	4.1	0	27	32	30
Cellulose powder	20	β-1,4	24	4.1	0	13	42	32
Avicel	20	β-1,4	24	4.1	0	18	80	40
Filter paper	20	β-1,4	24	4.1	0	24	108	85
Alkaline-swollen cellulose	2	β-1,4	24	4.1	0	3	6	24
CMC	6.75	β-1,4	0.5	0.8	0	21	0	315
p-Nitrophenyl-β-D-glucoside	0.24		0.5	0.8	671*	0	0	0
Cellobiose	2	β-1,4	0.5	0.28	135	0	0	0
Glycol chitosan	2	β-1,4 (glucosamine)	24	4.1	0	0	0	0

Mannan	2	β-1,4 (Mannose)	24	4.1	0	0	0	0
Xylan	2	β-1,4 (Xylose)	0.5	4.1	146	76	0	0
Chitin	2	β-1,4	24	4.1	0	0	0	0
Cellulodextrins (D.P. 3 - 6)	2	β-1,4	24-4	4.1	+	+	+	+
Polygalacturonic acid	2	α-1,4	24	4.1	0	0	0	0
Lichenan	2	β-1,4; β-1,3	0.5	1.65	132	41	3	38
Laminarin	2	β-1,3; β-1,6	6	4.1	+	+	0	0
CM-pachyman (D.S. 0.32)	2	β-1,3; β-1,6	0.5	4.1	76	65	0	0
Yeast glucan	2	β-1,3; β-1,6	24	4.1	105	205	0	0

Substrates were incubated with enzyme at 60°C for 24 h incubation and 67°C for 0.5 h incubation under the conditions defined. Activity was measured by the appearance of reducing sugars except for *p*-nitrophenyl-β-D-glucoside and cellobiose which were tested as described in Materials and Methods. Hydrolysis of laminarin and cellulodextrins was monitored using Sephadex G-15 gel filtration because of the high reducing power of the blanks. +, reducing groups formed: quantitative data not obtained. * as μg *p*-nitrophenol.

the same period of time.

The results as shown in Table 5 revealed that the three isolated cellulases exhibited varying degrees of degrading ability against the native celluloses tested. Filter paper was the most amenable to degradation. Both the acid- and alkaline-swollen cellulose were readily hydrolysed although the substrates are insoluble. As with the soluble CMC, a synthetic polymeric substrate, an interesting result was obtained. Cellulase II, which was found to be particularly active against native celluloses, had no hydrolytic activity toward CMC even at a relatively high protein concentration (6.25 µg/ml). Indeed, 94% of the CMC hydrolysing activity was confined to the cellulase III component.

A noteworthy difference in the specificity of these cellulase preparations was that cellulase I, unlike the other two cellulases, readily hydrolysed the native, mixed β-1,3; β-1,6 polysaccharides such as yeast-glucan, laminarin and CM-pachyman. With a yeast-glucan substrate concentration of 0.2% ($^{w}/_{v}$), the production of reducing and groups could be detected from the action of 6 ng enzyme/ml, which is an enzyme concentration an order of magnitude less than that used with CMC as substrate. The pH activity profiles for β-1,4 and β-1,3; β-1,6 substrates were compared. It was found that the pH-activity profile with respect to CMC showed the same sharp pH optimum at pH 4.5 as that obtained for yeast-glucan (Figure 28). In addition, the pH and temperature-stability profiles (Figures 29, 30) of the enzyme assayed on the two different substrates were also similar. The temperature optimum for activity (Figure 27) on yeast-glucan was found to be 65°C compared with than on

CMC at 75°C.

Lichenan (2 mg/ml), the only mixed β-1,4; β-1,3 polysaccharide tested, proved to be susceptible to degradation by all the three cellulase enzymes. This mixed-glucan-hydrolysing activity was also found by Hurst and his co-workers (1978) to be common to all the three cellulase bands isolated by disc-gel electrophoresis from *A. niger*.

Of the non-glucosidic polymers tested, xylan was the only polysaccharide degraded. It was only degraded by cellulase I enzyme. The specific activity of the xylanase was comparable with the specific activity of the CM-pachymanase.

As might be expected, β-glucosidase had no hydrolytic activity toward native, insoluble cellulose as well as soluble CMC. It was active against *p*-nitrophenyl-β-D-glucoside and cellobiose and even more active against xylan, lichenan and CM-pachyman than cellulase I. The possibility of this mixed-glucan-hydrolysing activity being a contaminant of the cellulase I component did not account for the absence of any cellulolytic activity exhibited by the β-glucosidase component, thus indicating that these activities of β-gluco sidase are unlikely to be due to a contaminant of cellulase I.

The purified enzymes were devoid of chitinase (or lysozyme like) and pectinase activity.

Thus, from the results obtained, it was noted that a distinguishing feature of each of the present four enzymic components is a characteristic action on their particular substrate. Although all three cellulase components showed varying degrees of ability in hydrolysing native, insoluble

cellulose, one of the components, cellulase II, could not degrade the soluble CMC which was hydrolysed by the other two components. The latter two components differed from each other in that one of them, cellulase I, could hydrolyse mixed β-1,3; β-1,6 polysaccharides while the other could not. None of the cellulase components had activity on either *p*-nitrophenyl-β-D-glucoside or cellobiose which were hydrolysed by the β-glucosidase component.

Other properties of the purified enzymes

The properties of the purified β-glucosidase and cellulase enzymes were studied on the substrates on which the enzyme was most active (Table 5). Thus, β-glucosidase was assayed on *p*-nitrophenyl-β-D-glucoside, cellulase I on CMC and yeast-glucan, cellulase II on filter paper and cellulase III on CMC. Standard assay procedures were as described under Materials and Methods unless otherwise stated.

Temperature Optimum

Experiments were conducted in the temperature range 30 - 90^{o}C and with incubation times varying between 0.5 h and 24 h. Substrates were prepared in citrate-phosphate buffer, pH 5.0. CMC was prepared in the same buffer at pH 4.5. Both enzyme solution and substrate were heated to the requisite temperature for 5 min. before mixing together.

As shown in Figure 27, optimum temperatures for β-glucosidase and cellulolytic activities fell within the range of 60 - 75^{o}C. The highest optimum temperature in the

Figure 27. Effect of Temperature on the Activities of β-glucosidase and Cellulases.

Standard assays systems described under Materials and Methods were used except that the incubation temperatures were varied. Amount of enzymes used in each assay were 0.14 μg for β-glucosidase; 2.4 and 0.6 μg for Cellulase I with CMC and yeast-glucan as substrates, respectively; 6.25 μg for Cellulase II and 0.16 μg for Cellulase III. Other details were mentioned in the text. Points were the average of two determinations.

◆—◆ Cellulase I on CMC (substrate)

◇—◇ Cellulase I on yeast-glucan (substrate).

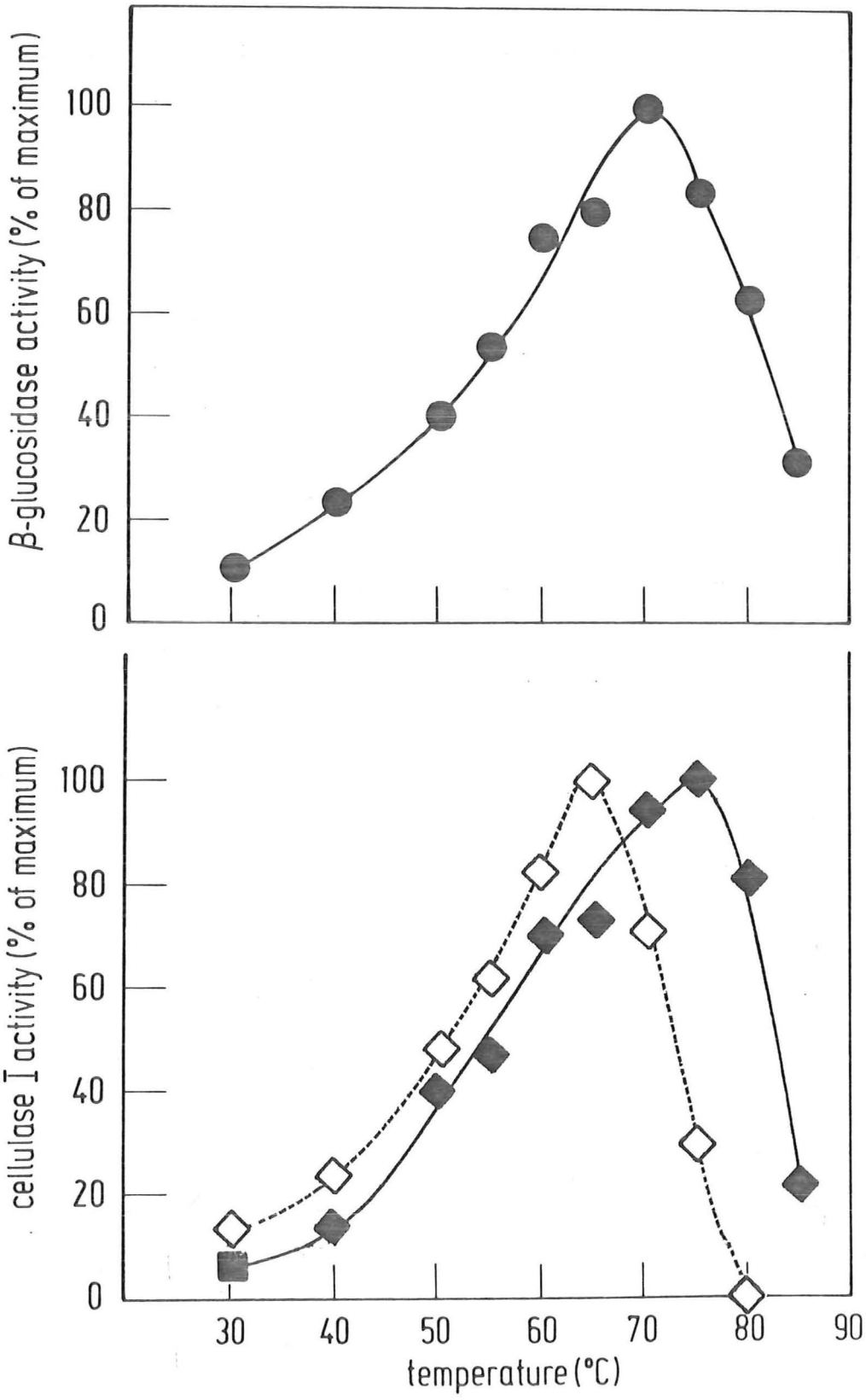
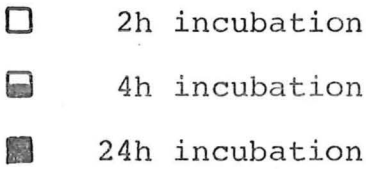
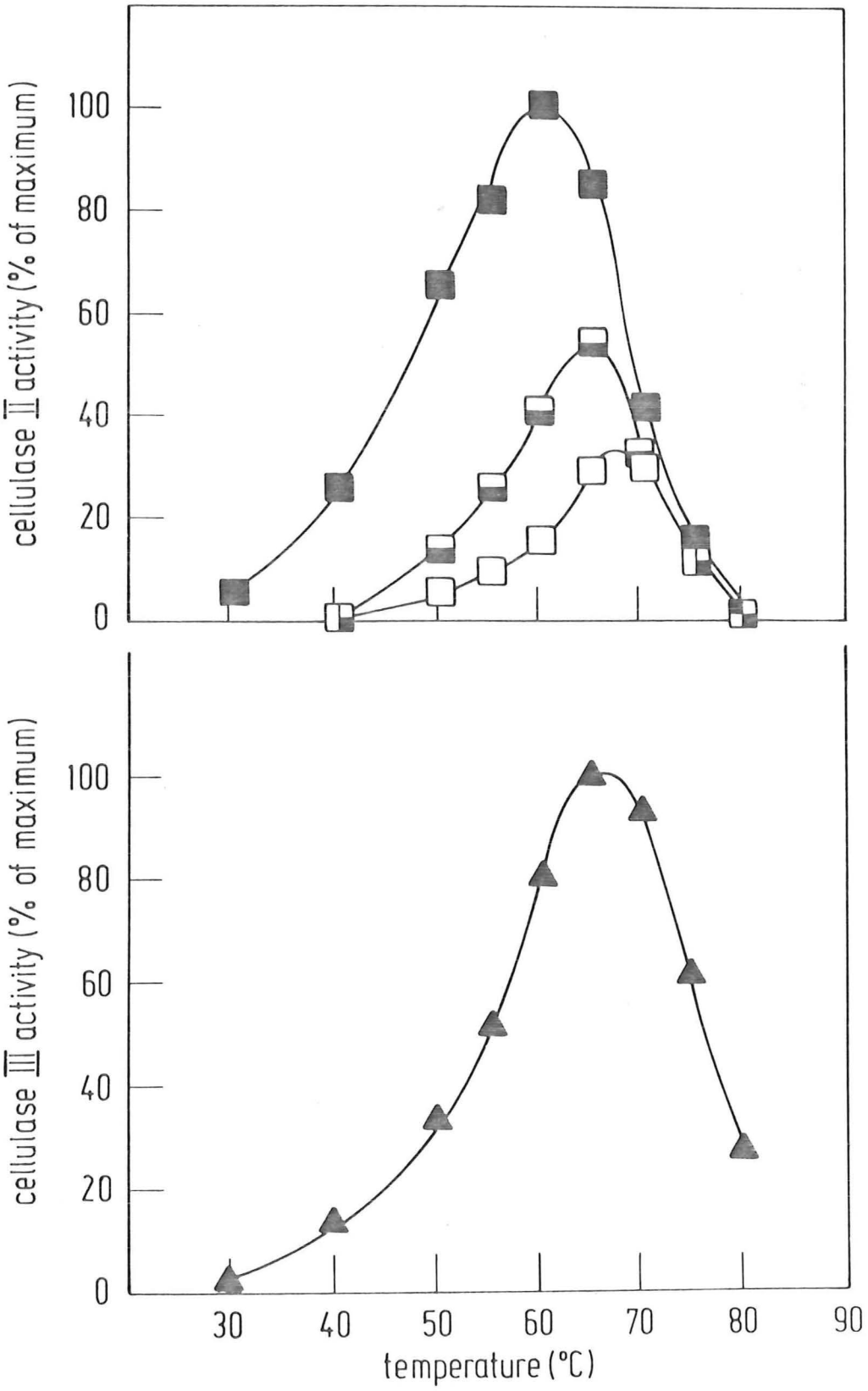

β-glucosidase activity (% of maximum)
100
80
60
40
20
0
cellulase I activity (% of maximum)
100
80
60
40
20
0
30
40
50
60
70
80
90
temperature (°C)

Figure 27. Continued




cellulase II activity (% of maximum)
100
80
60
40
20
0
cellulase III activity (% of maximum)
100
80
60
40
20
0
30
40
50
60
70
80
90
temperature (°C)

test was observed with cellulase I acting on CMC (75°C), whereas hydrolysis of yeast-glucan by the same enzyme was most efficient at 65°C. The difference observed in the temperature optimum on the two substrates could be due to inactivation of the enzyme acting on yeast-glucan at high temperatures with the long period of incubation (24 h). On CMC, a much shorter period of incubation (0.5 h) was used. The optimum temperature for cellulase II acting on filter paper dropped from 2 h to 24 h. Activity on *p*-nitrophenyl-β-D-glucoside was optimal at 70°C. Cellulase III showed a temperature optimum at 65°C with CMC as substrate (Figure 27). This is slightly lower than that recorded with the crude enzyme preparation (Figure 9). Activities at 30°C and below were low. At a temperature of 85°C with an incubation period of 0.5 h, β-glucosidase, cellulase I (on CMC) and cellulose III still retained about 20% of the optimum activity.

pH Optimum

The various enzymic activities were determined at various pH's in the range 3 - 9. The standard tests were used except that the substrate was prepared in either citrate-phosphate buffer or Tris buffer of the desired pH, and the enzyme preparations were suitably diluted with the same buffer. The pH of the reaction mixtures was measured prior to incubation. Further measurement at lower pH's was not made in order to avoid possible acid hydrolysis of the substrate. Incubation was carried out at the optimum temperature for activity of each enzyme: i.e., β-glucosidase

(70°C), cellulase II (60°C) and cellulase III (65°C). The activity of cellulase I on CMC and yeast-glucan was assayed at 75 and 65°C respectively.

As shown in Figure 28, hydrolysis of the various substrates by the purified enzymes was confined to acid media, being optimal at pH 4.5 - 5.0, thus following closely that previously determined with crude culture filtrates (Figure 10). Cellulase activities decreased rapidly at pH's below 3.5 and little or no activity remained at pH 3.0. A drop in all the activities also occurred above pH 5.0 with little or no activity at pH 7.0 except cellulase III which still retained 25% of its maximum activity. The pH-activity profiles of cellulase I on both the CMC and yeast-glucan were similar with a sharp pH optimum at 4.5. The pH optimum for the other enzymes was found to be 4.8 for β-glucosidase and cellulase III and 5.0 for cellulase II.

Thermal Stability

Enzyme solutions were held at the temperatures indicated for 1 h in a water bath in the absence of substrate. After cooling in running tap water (10°C), they were assayed in the standard manners at their optimum temperature.

The results (Figure 29) indicate that the cellulases were completely stable up to about 65°C but were very quickly denatured at higher temperatures. At 70°C, at least 40% of the optimum still remained. A further increase in temperature of only 5° to 75°C almost completely inactivated all the cellulases; about 12% of the cellulase I activity assayed on CMC remained. β-glucosidase was more thermostable than

Figure 28. Effect of pH on the Activities of β-glucosidase and Cellulases.

The enzymic activities were measured in the standard assay systems except that the buffer and pH were varied. Buffers used were citrate-phosphate (0.1M citric acid; 0.2M di-basic sodium phosphate) buffer (pH 3-7) and 0.2M Tris-HCl (pH 8, 9). Amount of enzymes used in each assay was as stated for Fig. 27. Points were the average of two determinations.

Cellulase I on CMC

Cellulase I on yeast-glucan.

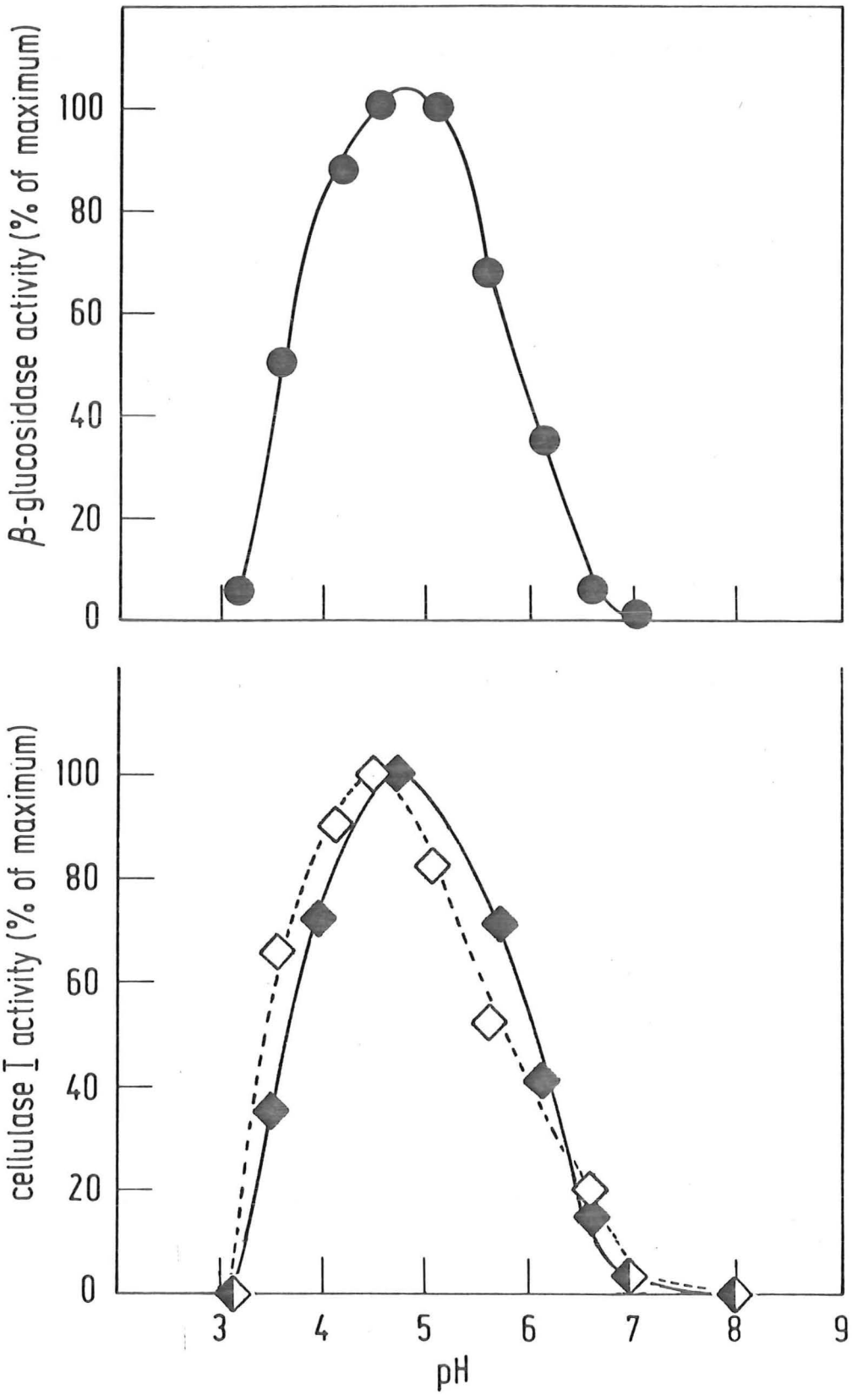

β-glucosidase activity (% of maximum)
100
80
60
40
20
0
cellulase I activity (% of maximum)
100
80
60
40
20
0
3
4
5
6
7
8
9
pH

Figure 28. Continued.

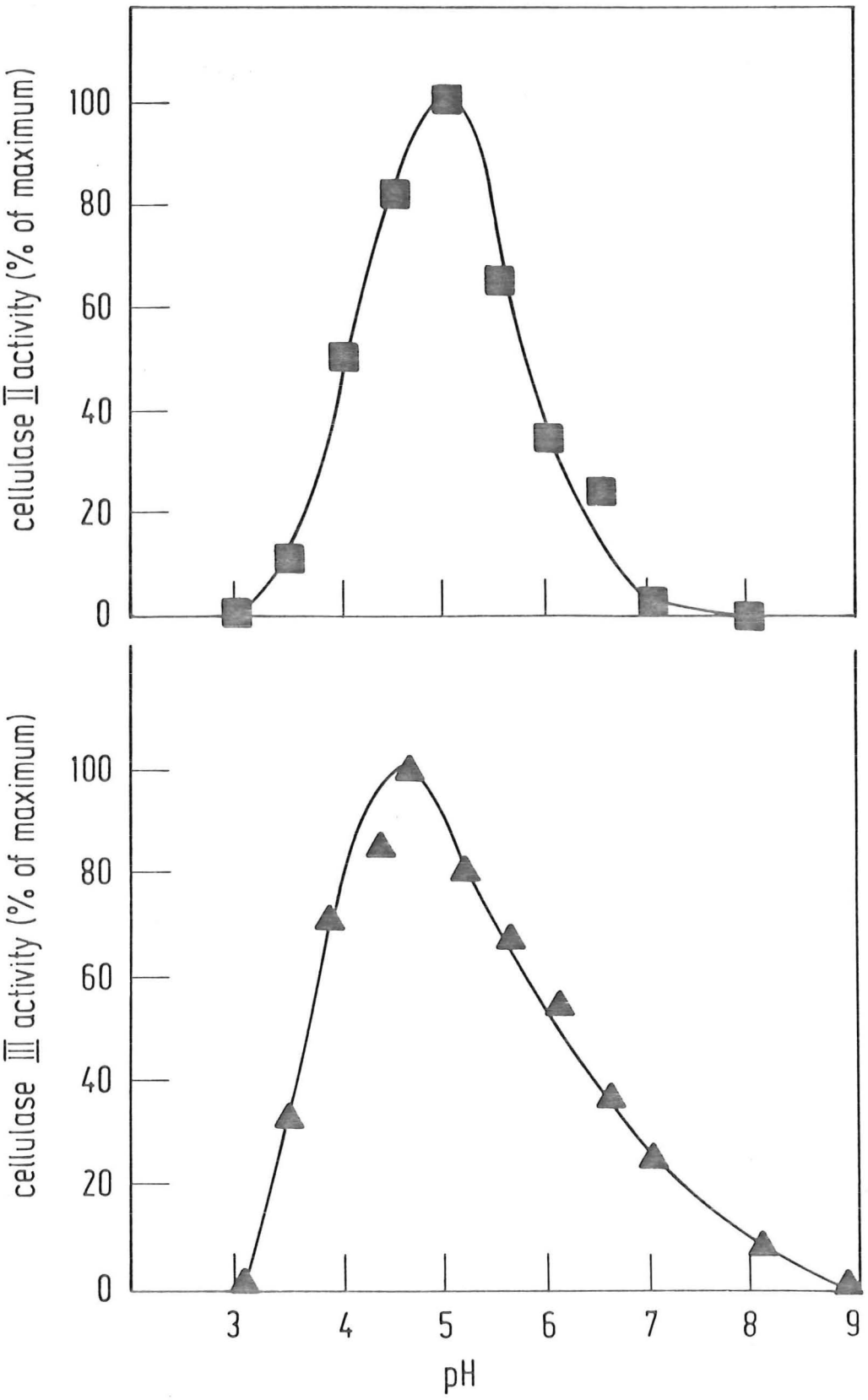
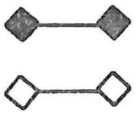

cellulase II activity (% of maximum)
100
80
60
40
20
0
cellulase III activity (% of maximum)
100
80
60
40
20
0
3
4
5
6
7
8
9
pH

Figure 29. Heat Stability of the β-glucosidase and Cellulase Enzymes.

Diluted enzyme solutions of same concentrations as those used in the temperature and pH optimum experiments were used. They were subjected to various temperatures indicated for 1 h. Activities were tested as usual at the optimum temperature and pH of each purified enzyme. Points were the average of two determinations.



Cellulase I on CMC

Cellulase I on yeast-glucan.

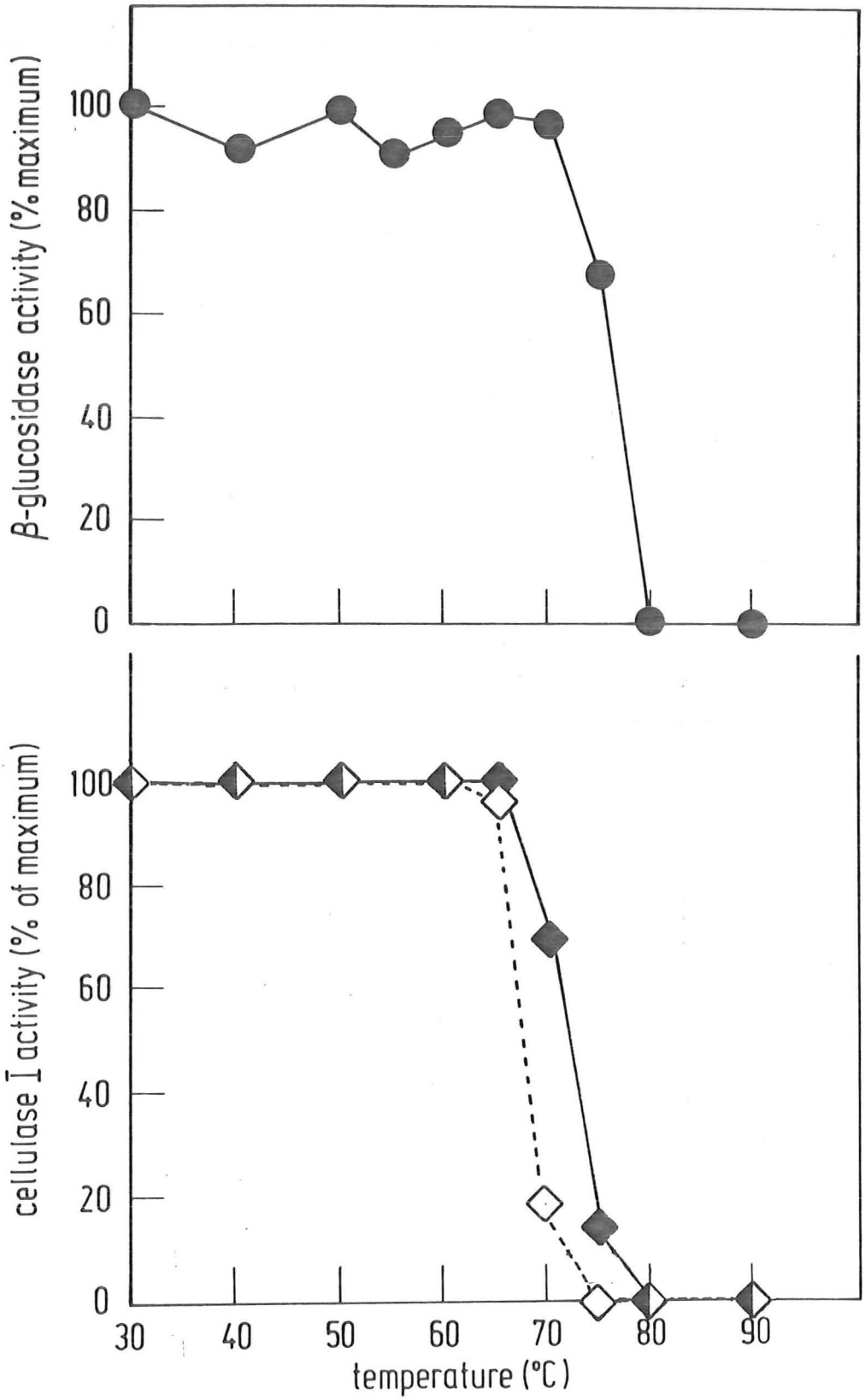
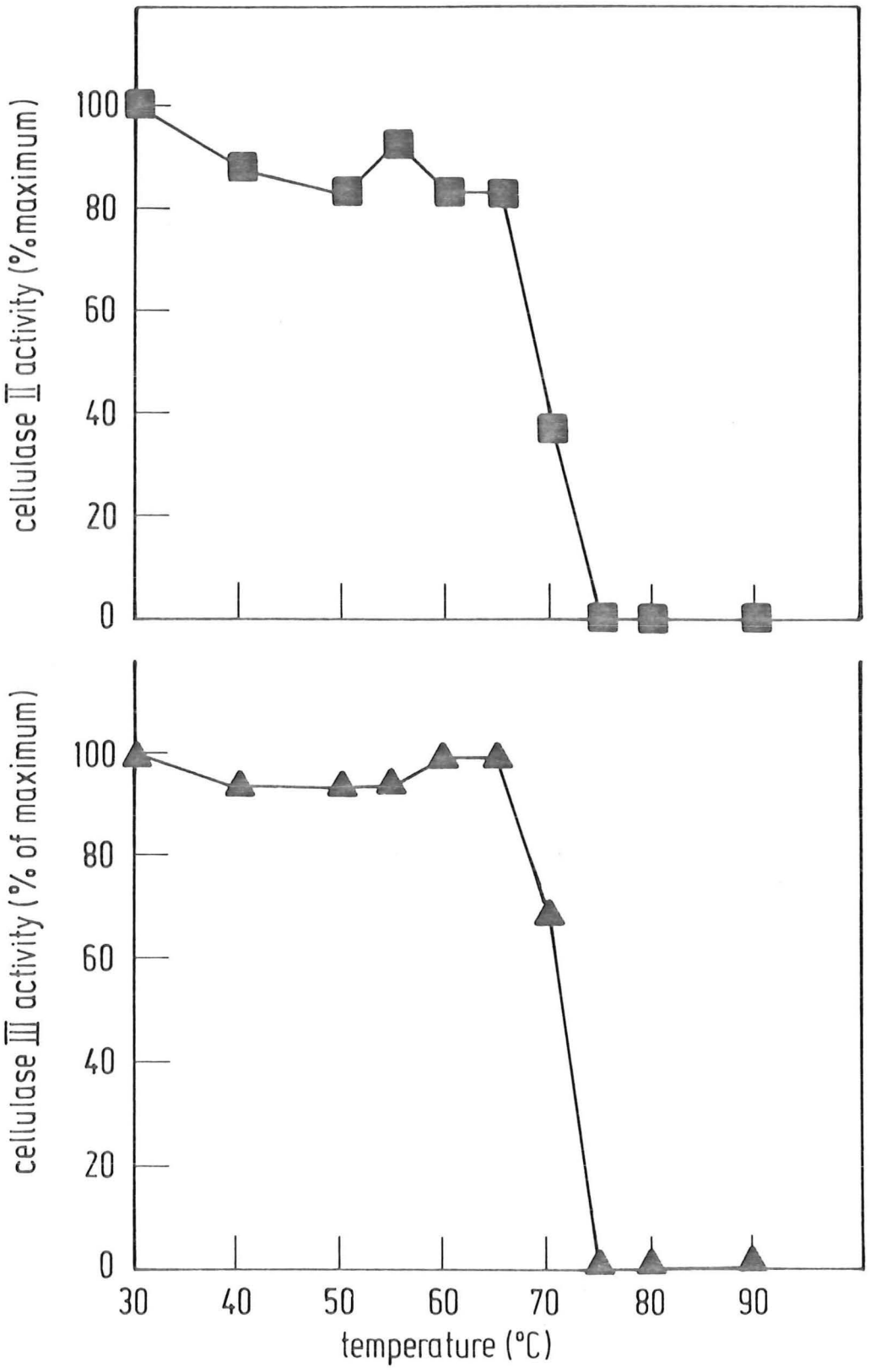

β-glucosidase activity (% maximum)
100
80
60
40
20
0
cellulase I activity (% of maximum)
100
80
60
40
20
0
30
40
50
60
70
80
90
temperature (°C)

Figure 29. Continued


cellulase II activity (% maximum)
100
80
60
40
20
0
cellulase III activity (% of maximum)
100
80
60
40
20
0
30
40
50
60
70
80
90
temperature (°C)

cellulases. The enzyme was totally stable up to 70°C and still retained 70% of its optimum activity at 75°C.

pH Stability

Figure 30 illustrates the effect of pH on the stability of β-glucosidase and cellulase enzymes.

In general, the pH stability curves of the enzymes are much broader than the pH activity curves obtained at the same temperature. All the enzymes were stable at pH 8.0, yet the activities at this pH were quite low. Cellulase II revealed marked stability to both acidic (pH 2) and alkaline (pH 12) condition. Optimum stability of all enzymes was shown at a pH range of 6 to 8.

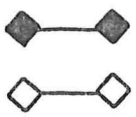
Enzyme Kinetics

Saturation curves of the purified enzymes acting on the corresponding substrate are shown in Figures 31 - 34. Table 6 summarises the K_m and v_{max} of the β-glucosidase and cellulases.

As different substrates were used for the enzymes in kinetic studies, care must be exercised in comparing their K_m and v_{max} values. From Table 6, it is noted that the K_m value for cellulase I acting on CMC is almost twice that obtained from cellulase III acting on the same substrate. The v_{max} for the above reactions clearly show that cellulase III is more efficient in hydrolysing CMC than cellulase I. The high K_m value for cellulase II on filter paper can possibly be attributed to the substrate being insoluble. Similarly on filter paper, the v_{max} is low which is indicative of the

Figure 30. pH Stability of the β-glucosidase and and Cellulase Enzymes.

Purified enzyme solutions were mixed with the appropriate buffers to give the required final pH and incubated at 25°C for 24 h. Substrates prepared in double-strength assay buffers were then added and the activities determined at the optimum temperature and pH of the purified enzymes. The amount of enzyme used in each assay was as stated for Fig. 27. The pH of each assay was checked after adding the substrate. Buffers used were: 0.4M KCl-HCl (pH 1.0, 2.0); 0.2M citric acid: 0.1M di-basic sodium phosphate (pH 3.0, 4.0, 5.0, 6.0, 7.0); 0.05M $Na_2B_4O_7.10\ H_2O$; 0.2M NaOH (pH 10.0, 11.0); 0.4M KCl-NaOH (pH 12.0, 13.0). Points were the average of two determinations.



Cellulase I on CMC

Cellulase I on yeast-glucan.

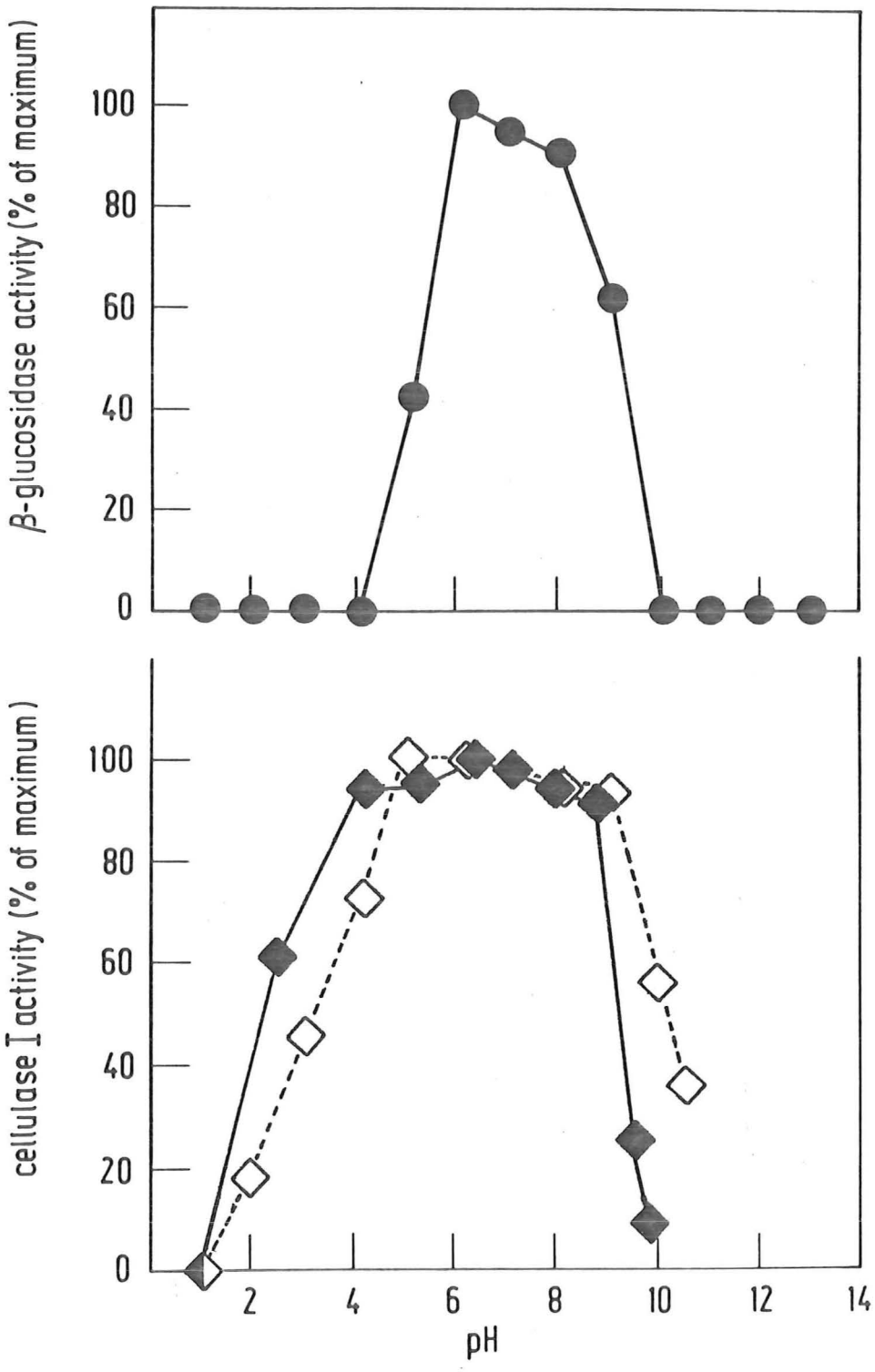

β-glucosidase activity (% of maximum)
100
80
60
40
20
0
cellulase I activity (% of maximum)
100
80
60
40
20
0
2
4
6
8
10
12
14
pH

Figure 30. Continued

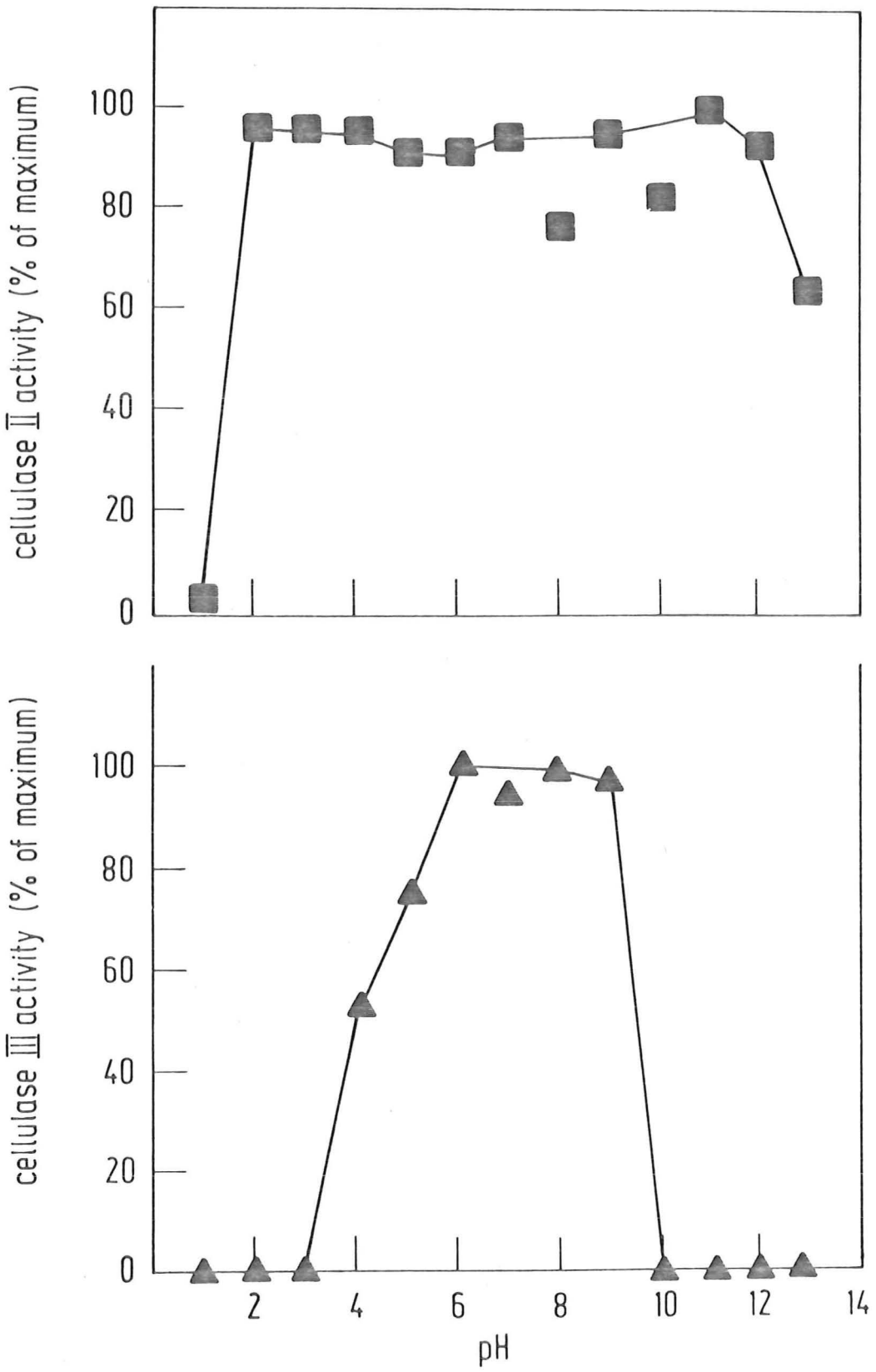
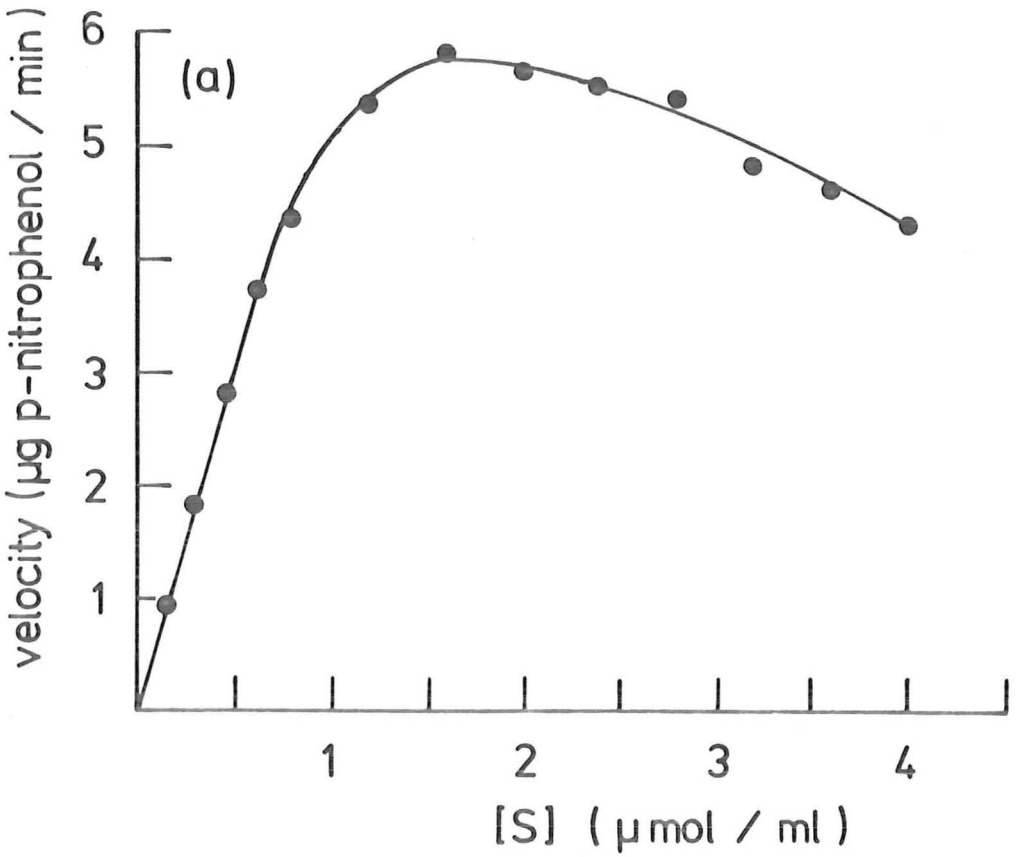

cellulase II activity (% of maximum)
100
80
60
40
20
0
cellulase III activity (% of maximum)
100
80
60
40
20
0
2
4
6
8
10
12
14
pH

Figure 31. Determination of K_m and V_{max} of β-glucosidase on ρ-nitrophenyl-β-D-glucoside.

(a) Saturation curve of β-glucosidase on ρ-nitrophenyl-β-D-glucoside. 0.14 µg of the enzyme was assayed under standard conditions at pH 4.8 and 70°C with varying substrate concentrations. Points were the average of two determinations.

(b) Double reciprocal plot. The data from Fig. 31a were replotted as reciprocals.


(a)
velocity (µg p-nitrophenol / min)
6
5
4
3
2
1
1
2
3
4
[S] (µ mol / ml)

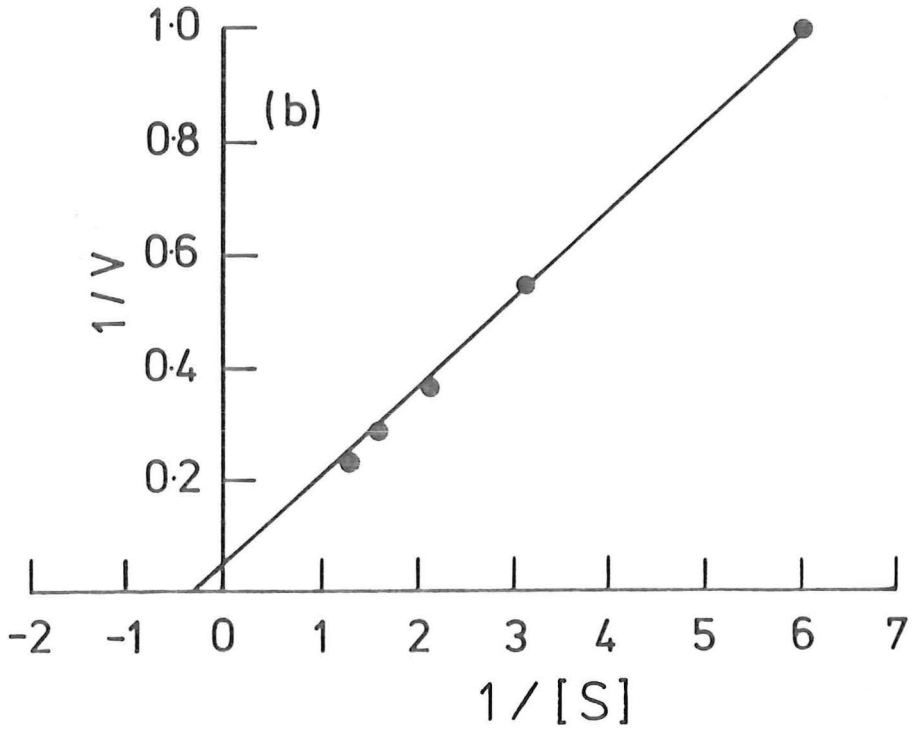

(b)
1·0
0·8
0·6
0·4
0·2
1 / V
-2
-1
0
1
2
3
4
5
6
7
1 / [S]

Figure 32. Determination of K_m and V_{max} of Cellulase I on CMC and Yeast-glucan

(a),(c) Saturation curves of Cellulase I on CMC (◆) and yeast-glucan (◇). The amount of enzyme used in each assay was 2.4 and 0.6 µg, respectively. Activities were assayed in the standard reaction mixture. CMC'ase was assayed at 75°C for 30 min and the activity on yeast-glucan at 65°C for 24h. Points were the average of two determinations.

(b), (d) Double reciprocal plots. The data from Fig. 32 a, c were replotted as reciprocals.

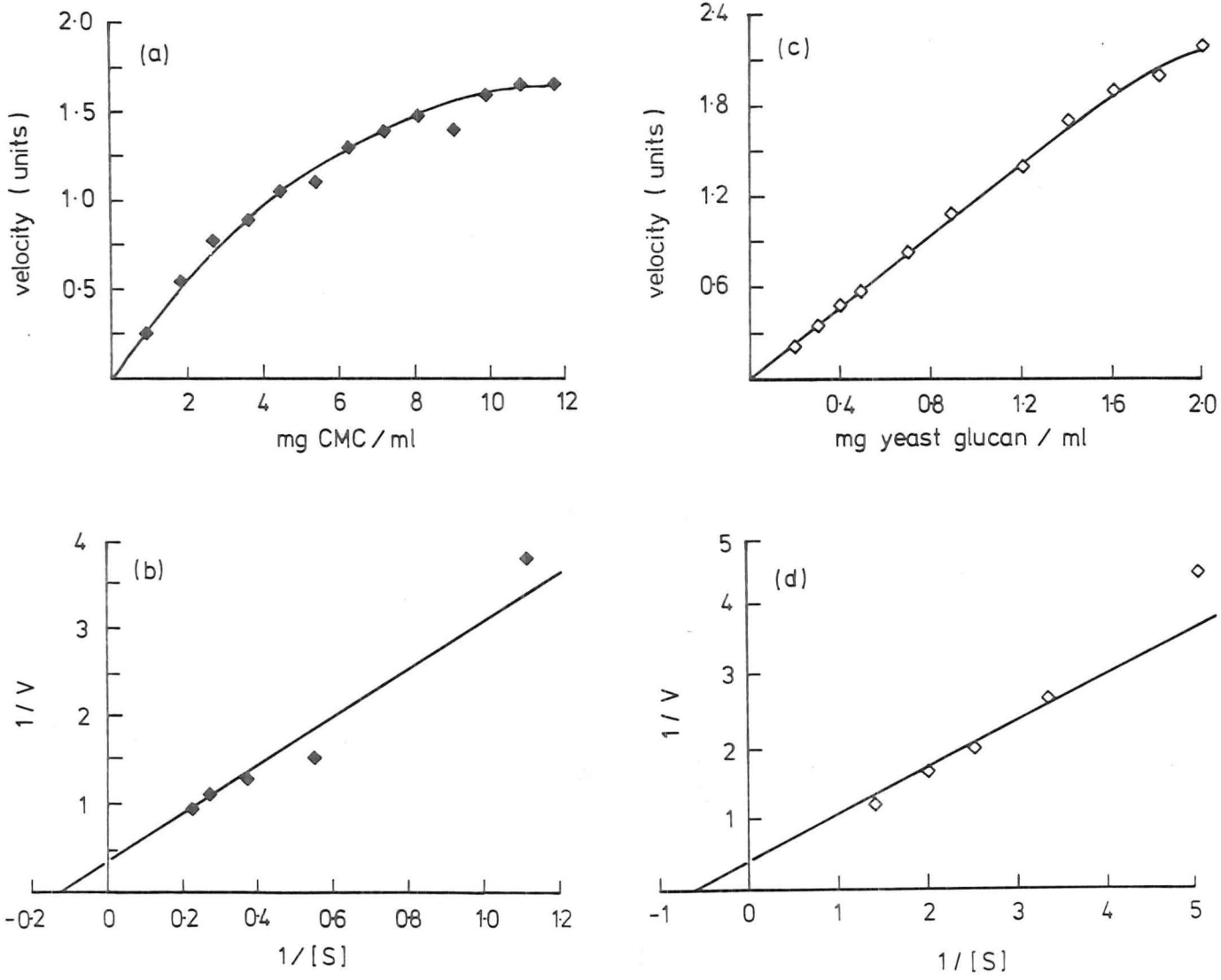
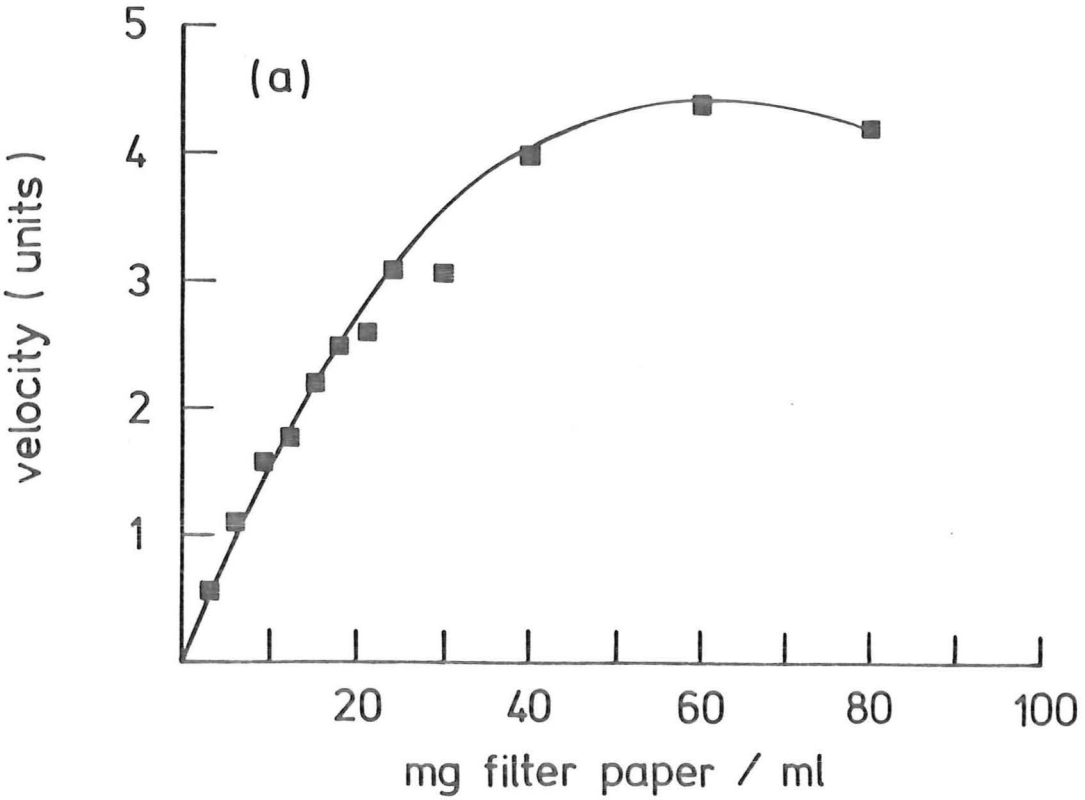

(a)
velocity (units)
2·0
1·5
1·0
0·5
2
4
6
8
10
12
mg CMC / ml
(c)
velocity (units)
2·4
1·8
1·2
0·6
0·4
0·8
1·2
1·6
2·0
mg yeast glucan / ml
(b)
1 / V
4
3
2
1
-0·2
0
0·2
0·4
0·6
0·8
1·0
1·2
1 / [S]
(d)
1 / V
5
4
3
2
1
-1
0
1
2
3
4
5
1 / [S]

Figure 33. Determination of K_m and V_{max} of Cellulase II on Filter Paper.

(a) Saturation curve of Cellulase II on filter paper. 6.25 µg of the enzyme were assayed at pH 5.0 with varying concentrations of the substrate in the standard assay system. Duplicate assays were carried out.

(b) Double reciprocal plot. The data from Fig. 33a were replotted as reciprocals.


(a)
velocity (units)
5
4
3
2
1
20
40
60
80
100
mg filter paper / ml

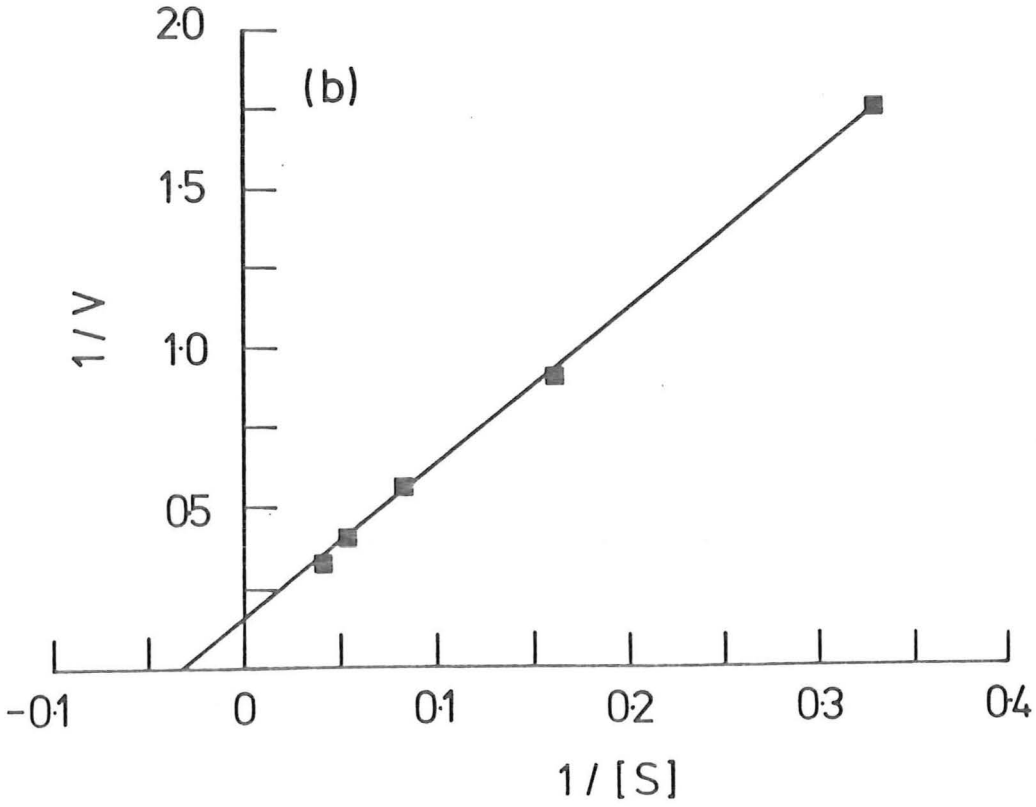
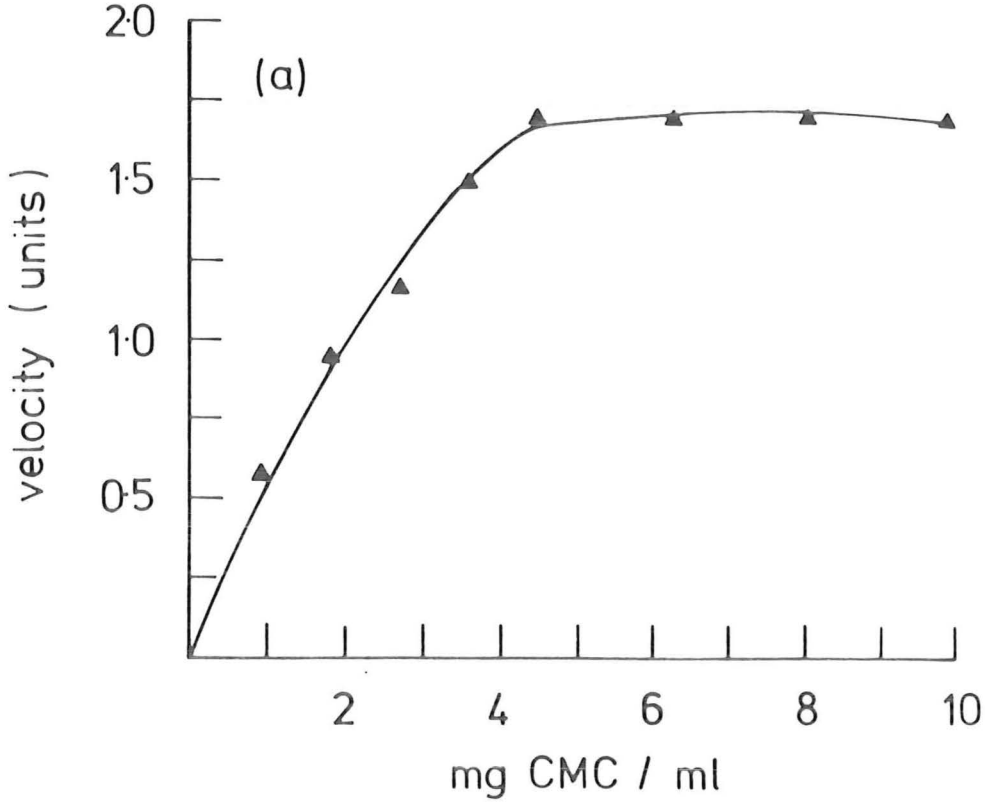
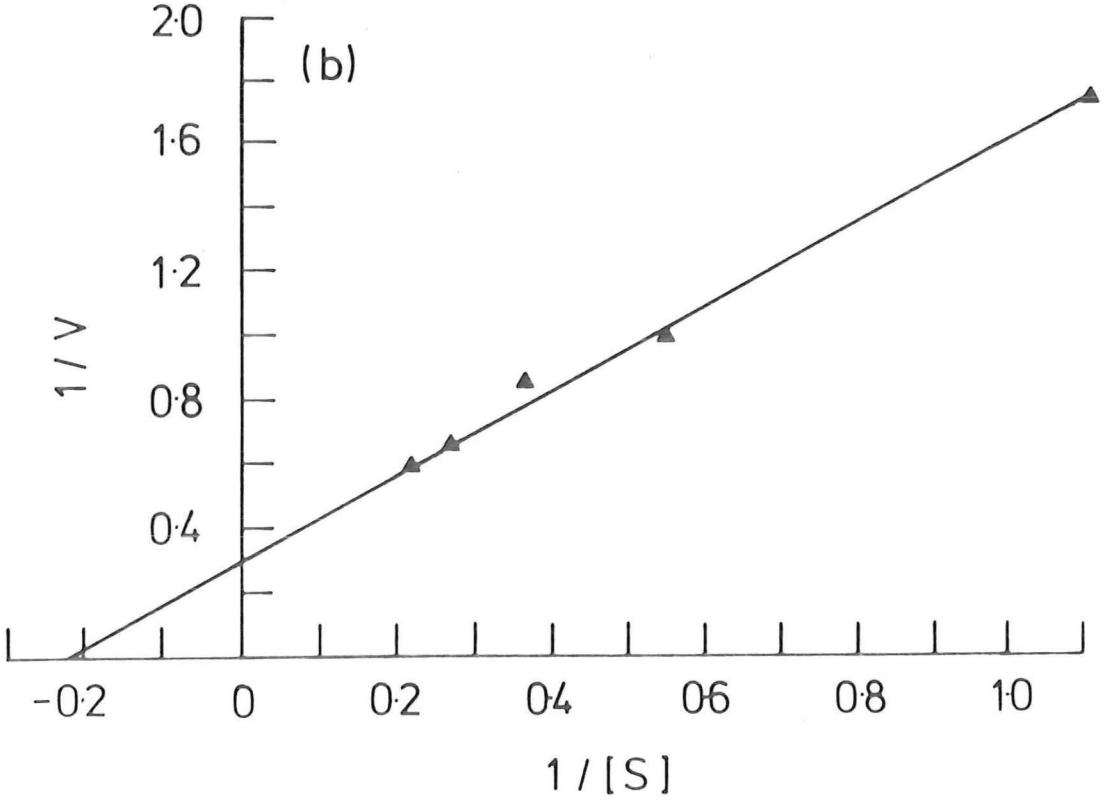

(b)
2·0
1·5
1·0
05
1 / V
-0·1
0
0·1
0·2
0·3
0·4
1 / [S]

Figure 34. Determination of K_m and V_{max} of Cellulase III on CMC

(a) Saturation curve of Cellulase III on CMC. 0.16 µg of the enzyme was assayed at pH 4.5 with varying concentrations of CMC. Standard assays were carried out at 70°C for the purified enzyme. Each point represents the average of two determinations.

(b) Double reciprocal plot. The data from Fig. 34a were replotted as reciprocals.


(a)
velocity (units)
2·0
1·5
1·0
0·5
2
4
6
8
10
mg CMC / ml


(b)
1 / V
2·0
1·6
1·2
0·8
0·4
-0·2
0
0·2
0·4
0·6
0·8
1·0
1 / [S]

difficulty in degrading the insoluble cellulose by the enzyme. Cellulase I was found to cleave β-1,4 linked CMC more readily than the mixed β-1,3; β-1,6 linked yeast-glucan.

TABLE 6 K_m and ν_{max} of β-glucosidase and cellulases

Enzyme	K_m (mg substrate/ml)	ν_{max} (units/µg protein) x 10^3
β-glucosidase (on *p*-nitrophenyl-β-D-glucoside)	1.03	1200.0
Cellulase I (on CMC)	8.86	44.3
Cellulase I (on yeast-glucan)	1.78	2.0
Cellulase II (on filter paper)	34.41	0.8
Cellulase III (on CMC)	4.68	722.0

K_m and ν_{max} values were determined under conditions described in Figures 31 - 34, by the procedures outlined in Materials and Methods(ii).

Enzyme Synergism

Synergism between purified cellulases of a number of fungi has been reported by a number of workers (Olutiola & Ayers, 1973; Wood, 1968; Selby & Maitland, 1967).

The activities of the purified enzyme components towards filter paper were examined singly and in various combinations such that the original cellulase complex was reconstituted in a stepwise manner (Table 7). Release of reducing sugars from the cellulose was used as an indicator of enzymic activity.

TABLE 7 Cellulolysis of native cellulose (filter paper) by purified components alone and in combination

Enzyme	Soluble reaction product (glucose equivalent; µg/ml)	Expected value if no synergism	Increase in products (%)
β-glucosidase	0		
CI	30		
CII	75		
CIII	73		
β-glucosidase + CI	60	30	100
β-glucosidase + CII	160	75	113
β-glucosidase + CIII	132	73	81
CI + CII	120	105	14
CI + CIII	85	103	0
CII + CIII	170	148	14
β-glucosidase + CI + CII	230	105	119
β-glucosidase + CI + CIII	190	103	84
β-glucosidase + CII + CIII	280	148	89
CI + CII + CIII	176	178	0
β-glucosidase + CI + CII + CIII	340	178	91

Enzyme activity is expressed as glucose equivalent of reducing sugars (µg/ml) liberated from filter paper in 24 h at 60°C. The standard assay was used as described in Materials and Methods. The amount of each enzyme used in the assay system was 0.7, 2.4, 2.5 and 2.4 µg for β-glucosidase, CI, CII and CIII respectively. Each value is the mean of duplicates.

There was no synergism between any of the three cellulases CI, CII and CIII on filter paper; the slight increase in the reducing sugars formed between components CI & CII and CII & CIII was too small to be considered significant. Whenever the enzyme under test contained β-glucosidase, there was an increase in the absorbance reading in the reducing sugar assay. The presence of β-glucosidase caused an increase of between 80 to 120% over that of the expected value if no synergism occurred between components. This led to an investigation of the products formed from cellulose by components acting alone and in combination from the above experiments. Figure 35a shows that the major product of hydrolysis of filter paper by each of the cellulases acting alone was cellobiose. When acting in the presence of β-glucosidase, glucose was the final product. Thus, for each cellobiose molecule formed by the action of cellulase component on filter paper, two molecules of glucose were created by hydrolysis of β-glucosidase on the cellobiose. This resulted in a two-fold increase in the colorimetric estimation of the reducing sugars observed in the reaction mixtures containing β-glucosidase. As such, the results obtained from these experiments indicated that there is no significant synergism between the four purified cellulase and β-glucosidase components of *T. aurantiacus*.

Figure 35b shows that when the incubation period for each of the cellulase enzymes acting on filter paper was increased from 24 h to 48 h, glucose could be detected as a product. With acid-swollen cellulose as substrate, the hydrolytic products included some higher oligosaccharides. In the reaction mixtures containing β-glucosidase, glucose

Figure 35. Paper Chromatography of the Hydrolytic Products formed from Filter Paper and Acid-Swollen Cellulose by Cellulase Components Acting Alone and in Combination with β-glucosidase.

The reaction mixture (1.0 ml) containing 20 mg filter paper or acid-swollen cellulase in 0.9 ml citrate-phosphate buffer, pH 5.0 and 0.1 ml enzyme solution (concentration stated in Table 7) was incubated at 60°C with a drop of toluene added. Aliquots (0.1 ml) of the mixtures were chromatographed on paper and the products were analysed as described in Materials and Methods.

(a) On Filter Paper

Reaction time was 24 h. 1. standard cellobiose; 2. standard glucose; 3. mixture of CI, CII, CIII and β-glucosidase; 4. Cellulase II; 5. Cellulase I and 6. Cellulase III component.

(b) On Filter Paper

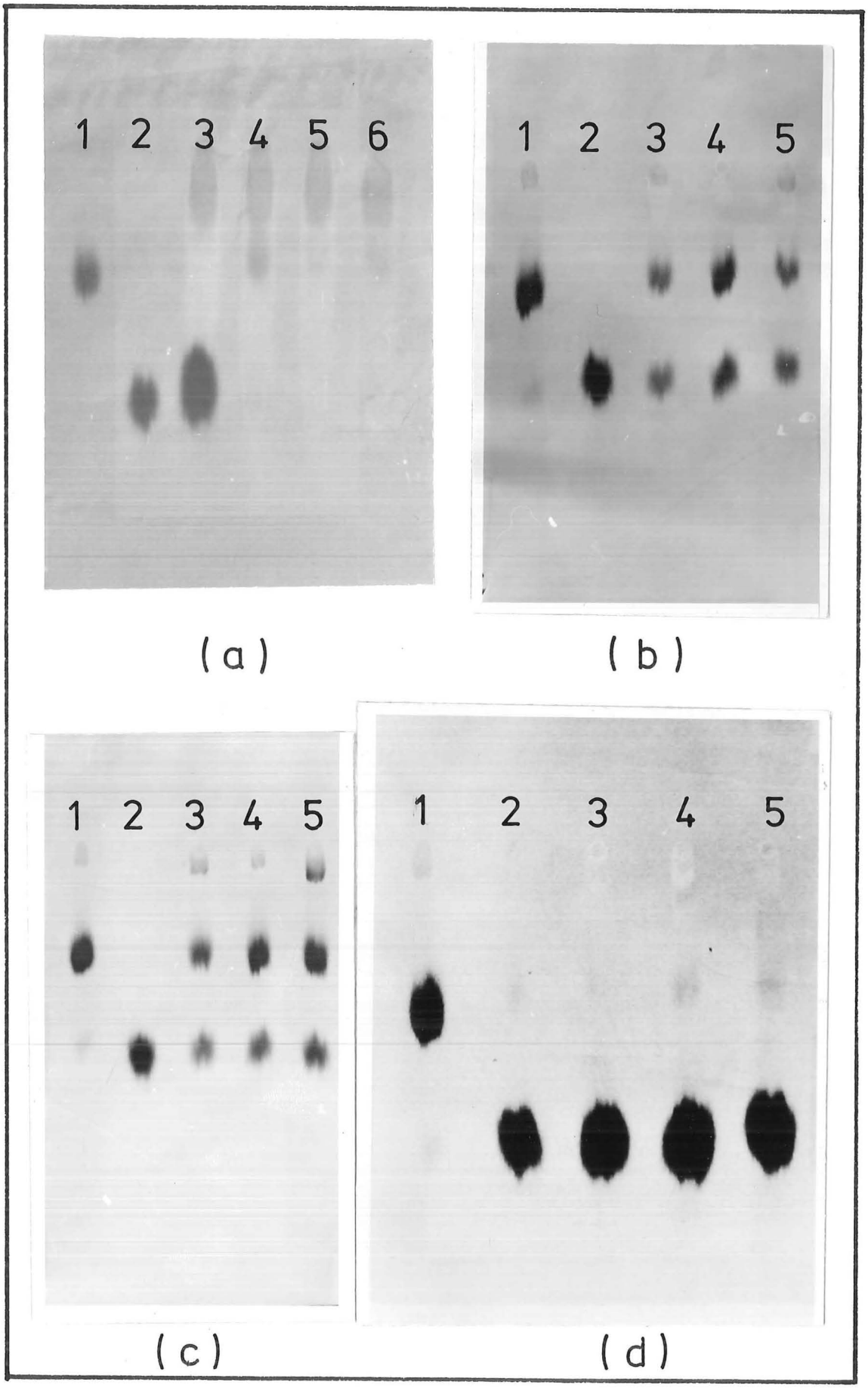
Reaction time was 48 h. 1. standard cellobiose; 2. standard glucose; 3. Cellulase I; 4. Cellulase II and 5. Cellulase III.

(c) On acid-swollen cellulose.

Reaction time was 24 h. Order as in (b).

(d) On Filter Paper

Reaction time was 24 h. Order as in (b) except that β-glucosidase was added in the reaction mixtures.


1 2 3 4 5 6
(a)
1 2 3 4 5
(b)
1 2 3 4 5
(c)
1 2 3 4 5
(d)

was the final product from filter paper (Figure 35d) and acid-swollen cellulose (not shown).

Nature of the enzymic action of cellulase III

CMC hydrolysis was followed by monitoring reducing end group production. No glucose was detected (Figure 36). An increase in reducing groups equivalent to 60 µg of glucose per ml of reaction mixture was formed after 30 min. incubation. Reactions mixtures which contained inactivated enzyme (1.6 µg/ml, immersion in a boiling water bath for 10 min.) exhibited little or no decrease in viscosity in 30 min. The presence of reducing groups was not detected in the heated enzyme reaction mixture. To further investigate the degradation specificity, the hydrolytic products taken at 10 min. intervals were chromatographed. Glucose and cellobiose were spotted as standards. The results in Figure 37 show the products to be cellobiose and other higher oligosaccharides. No glucose was identified, which indicated an endo- rather than exo-cellulolytic mode of action. This conclusion was supported by the observation that hydrolysis of CMC was accompanied by a rapid decrease in the viscosity of the solution (Figure 38).

The mode of action of cellulase III against cellulodextrins and reduced cellulodextrins

Information on the mode of action of some cellulases has been determined by using a series of β-1,4-linked oligosaccharides (Cole & King, 1964; Clarke & Stone, 1965; Pettersson, 1969; Streamer *et al.*, 1975).

Figure 36. Production of Reducing Sugar during the Hydrolysis of CMC by Cellulase III.

Each assay contained 0.1 ml enzyme solution (0.16µg) and 0.9 ml 0.75% (w/v) CMC in citrate-phosphate (0.1M citric acid; 0.2M di-basic sodium phosphate) buffer, pH 4.5 at 65°C. Tubes were removed from the incubation bath at times indicated and kept on ice. Reducing sugars (▲) and glucose (Δ) were determined as described in Materials and Methods.

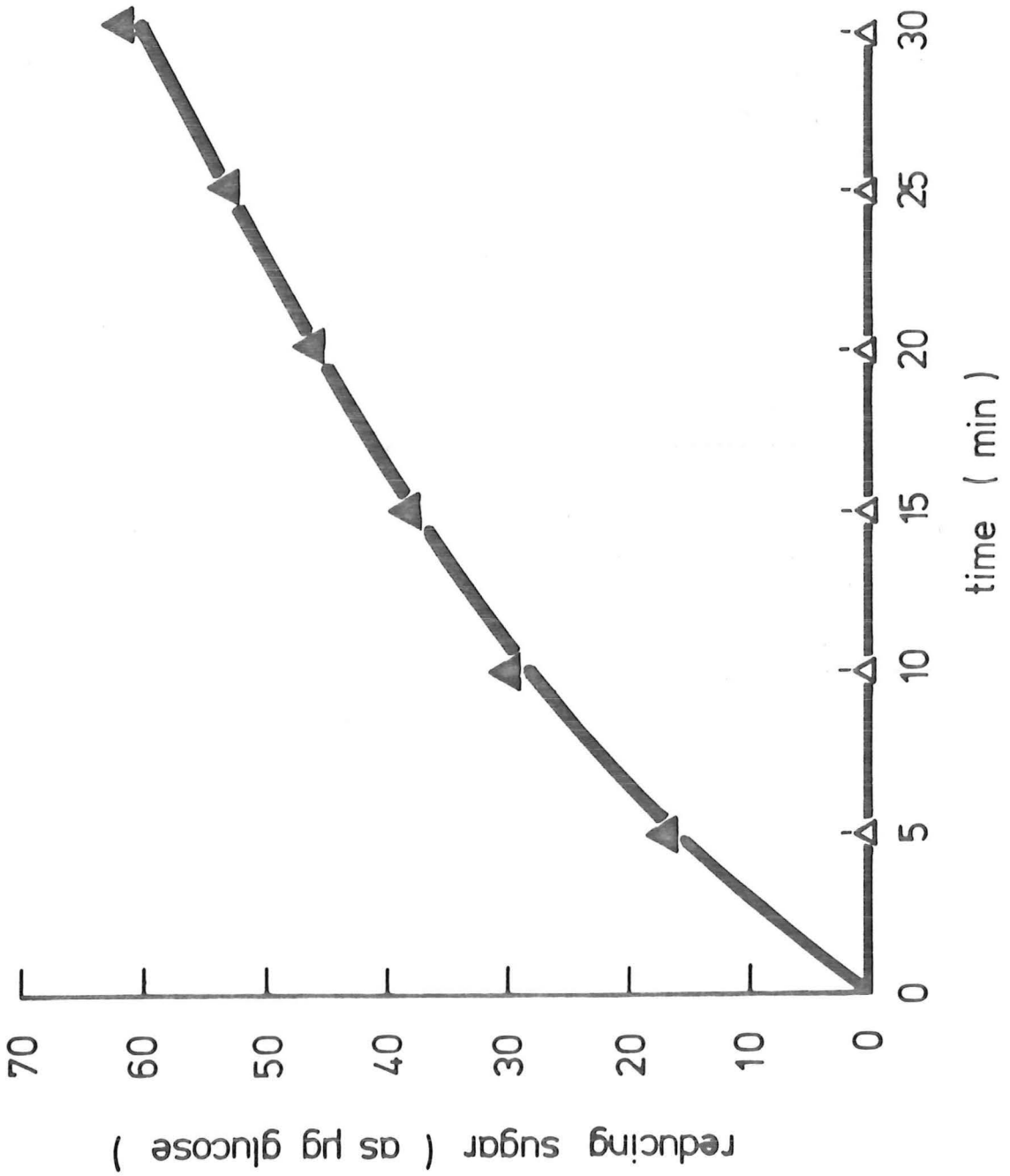

reducing sugar (as µg glucose)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
time (min)

Figure 37. Paper Chromatography of the Hydrolytic Products formed from CMC by the Cellulase III Component.

The reaction mixture (1.0 ml), containing 0.9 ml of 0.75% (w/v) CMC in the citrate-phosphate buffer, pH 4.5 and 0.1 ml enzyme solution (0.16 µg) was incubated at 65°C. Aliquots (0.1 ml) of the mixture removed were spotted onto Whatman No. 1 chromatography paper. Analysis of the products followed the procedures described in Materials and Methods. 1. 60 min. sample; 2. 30 min. sample; 3. 15 min. sample; 4. standard cellotriose, cellotetraose and cellopentaose and 5. standard glucose and cellobiose.

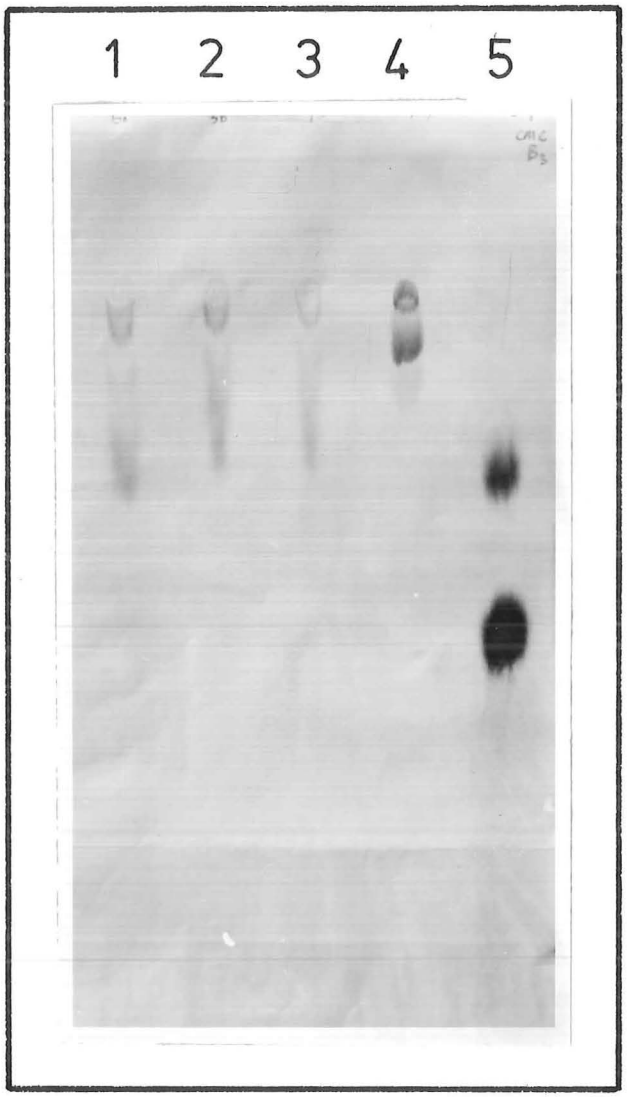
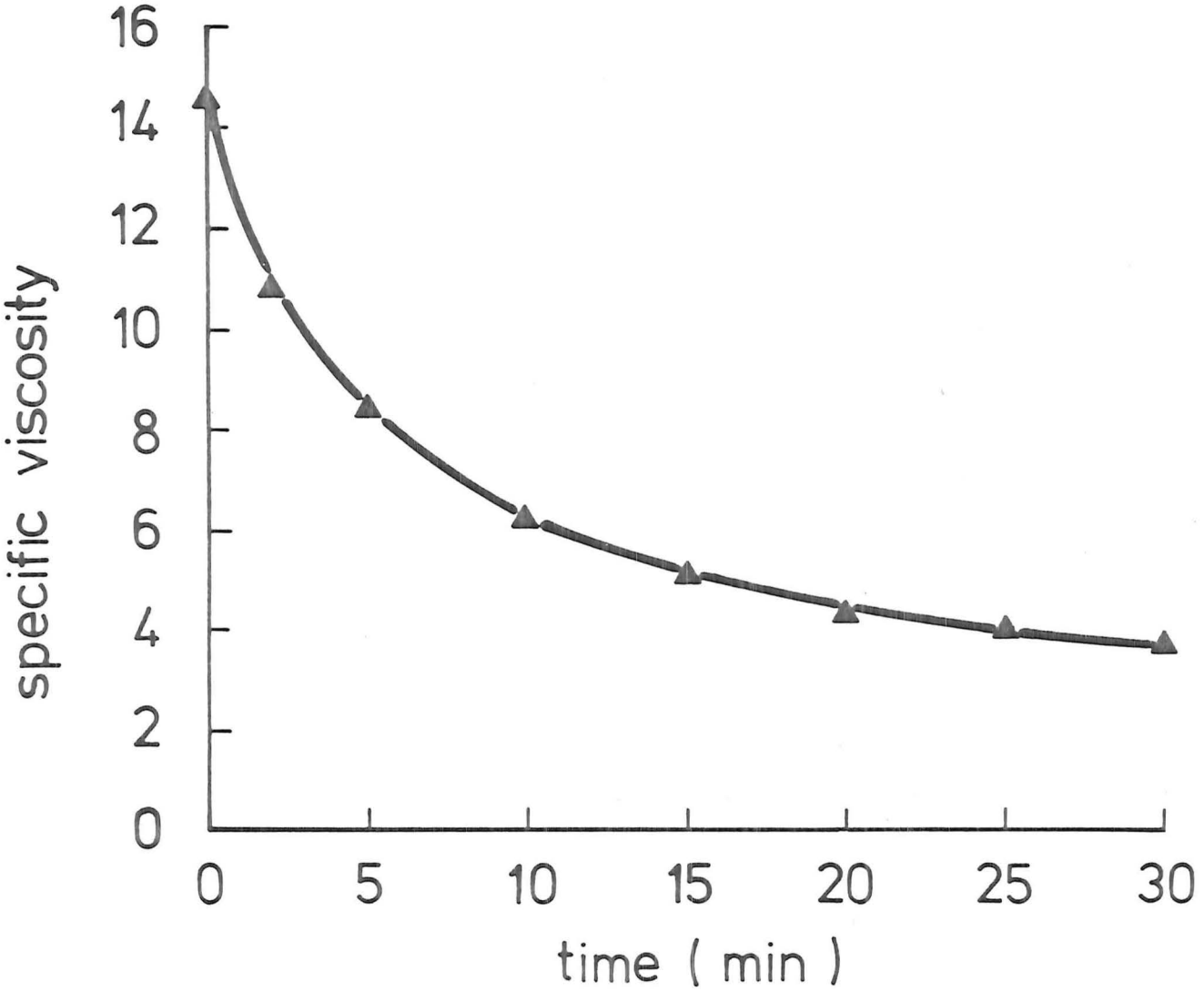

1
2
3
4
5

Figure 38. Decrease in Viscosity of CMC Solution by Cellulase III.

Five ml of 0.75% (w/v) CMC solution in citrate-phosphate buffer (0.1M citric acid; 0.2M di-basic sodium phosphate) pH 4.5 were mixed with 1.0 ml enzyme solution (0.041 µg) in a viscometer incubated at 65°C. The efflux time (sec.) of the mixture was taken at the times indicated. The specific viscosity at zero time was determined by substituting buffer for the enzyme solution.


specific viscosity
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
time (min)

The chemically reduced substrates permit reliable identification of the site of attack and where more than one bond is attacked, the relative frequencies of attack at each bond can be quantitatively determined. The location of the glycosyl bonds that are most susceptible to attack by cellulase III was ascertained by determining the change in the composition of the β-1,4 oligosaccharides and their reduced analogues by means of gel filtration. The hydrolysates were analysed on a column of Sephadex G-15 (1 x 85 cm) eluted with distilled water. In the reduced oligosaccharides, the terminal reducing glucose residue has been converted to a sorbitol residue by sodium borohydride. With the non-reduced substrate, cleavage at bond 1 and 5 or 2 and 4 (numbering from the non-reducing end of the molecule) yields identical products, and the bond cleaved cannot be identified. With the reduced substrate, for example cellohexaitol, cleavage at bond 1 would result in the production of glucose and cellopentaitol. Glucose, a reducing sugar, would be detected in the column eluate by the *p*-hydroxybenzoic acid hydrazide reagent, whereas the reduced oli-gosaccharide would not. Hence cleavage at bond 1 can be distinguished from cleavage at bond 5 as this latter hydrolytic pattern would yield cellopentaose and sorbitol. It was assumed that the sorbitol residue does not bias the action of the enzyme.

Figure 39a shows that cellotri-itol was hydrolysed with cellobiose as the main reducing sugar, some glucose was also produced. This indicated a preferential cleavage at bond 2 compared with bond 1.

Hydrolysis of cellotetraitol yielded cellotriose and cellobiose as major products with a trace of glucose

Figure 39. Column Chromatography of Reduced Cellulodextrins after Hydrolysis by Cellulase III.

Hydrolysates were analysed on a column of Sephadex G-15 (1 x 85 cm) eluted with distilled water at a rate of 10 ml/h. Fractions of 0.6 ml were collected and assayed for reducing sugars with PAHBAH reagent and total carbohydrate with anthrone-sulphuric acid reagent as described in Materials and Methods.

———— reducing sugars, A_{420} nm;

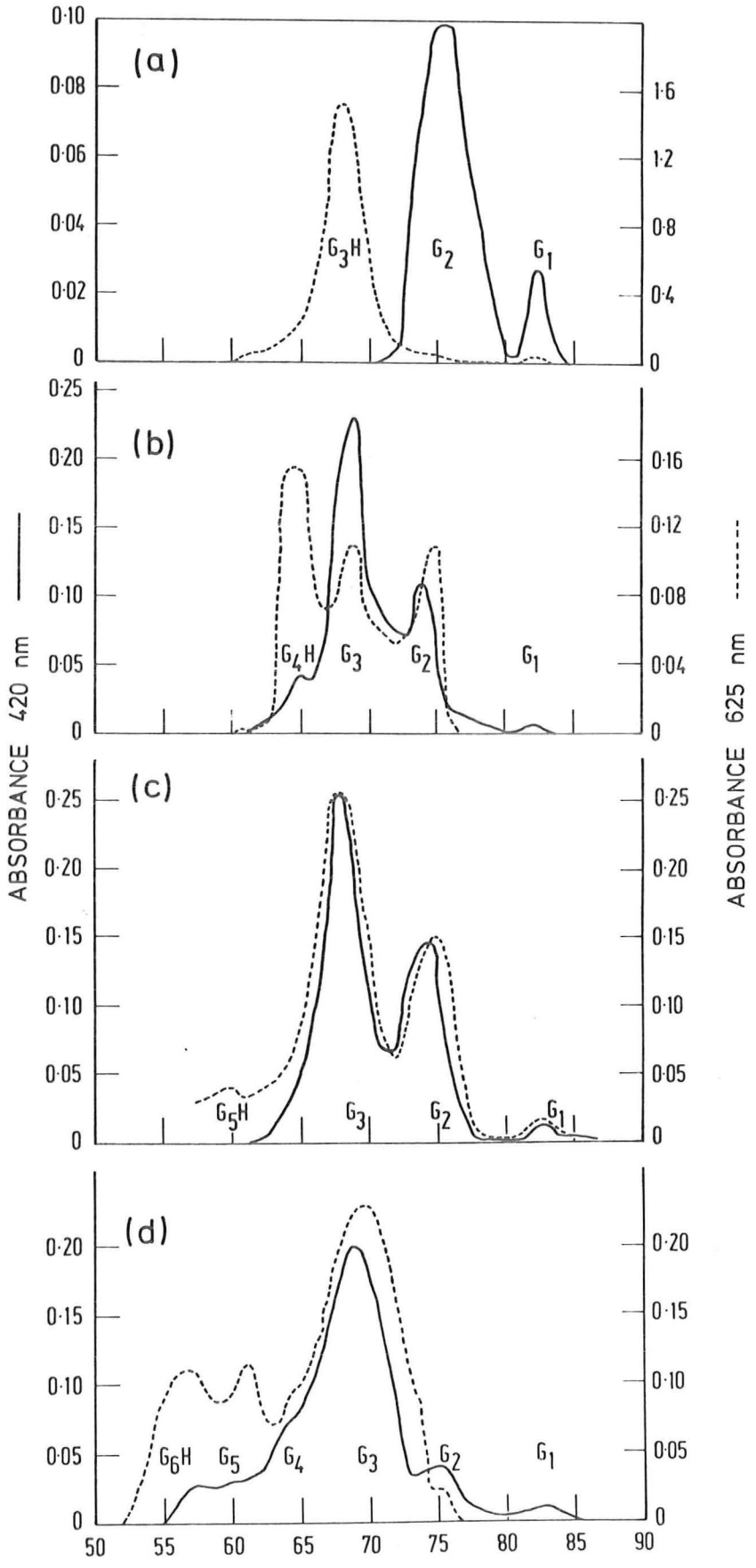
-------- total carbohydrate, A_{625} nm.

(a) Cellotri-itol hydrolysis: Incubation mixture contained 0.5 ml of cellotri-itol (1 mg/ml in citrate-phosphate buffer, pH 4.5) and 0.1 ml of enzyme (82.5 µg/ml). Time of hydrolysis at 60°C was 90h; reaction mixture was then heated for 5 min on a boiling water bath to inactivate the enzyme, cooled and then applied to the column.

(b) Cellotetraitol hydrolysis: Incubation mixture contained 0.5 ml of cellotetraitol, 1 mg/ml as in (a) and 0.1 ml of enzyme (82.5 µg/ml). Time of hydrolysis at 60°C was 12h; reaction mixture then treated as in (a).

(c) Cellopentaitol hydrolysis: Incubation mixture contained 0.5 ml of cellopentaitol, 1 mg/ml as in (a) and 0.1 ml of enzyme (8.25 µg/ml). Time of hydrolysis at 60°C was 2h; reaction mixture then treated as in (a).

(d) Cellohexaitol hydrolysis: Incubation mixture contained 0.5 ml cellohexaitol, 1 mg/ml as in (a) and 0.1 ml of enzyme (1.03 µg/ml). Time of hydrolysis at 60°C was 0.5h; reaction mixture then treated as in (a).


(a)
(b)
(c)
(d)
G_3H
G_2
G_1
G_4H
G_3
G_2
G_1
G_5H
G_3
G_2
G_1
G_6H
G_5
G_4
G_3
G_2
G_1
ABSORBANCE 420 nm
ABSORBANCE 625 nm
fraction no .

(Figure 39b), showing that bonds 2 and 3 were cleaved.

In the case of cellopentaitol, cellotriose was the predominant reducing sugar formed. Some cellobiose and trace amounts of glucose were also evident (Figure 39c), revealing that, although bonds 1, 2 and 3 are susceptible to cleavage, the penultimate glycosyl bond from the sorbitol end is the preferred cleavage point.

Hydrolysis of reduced cellohexaose gave cellotriose as major product. Other reducing sugars were also produced, but not in large amount (Figure 39d). Thus, again the central bonds in the glucose oligomer were the most susceptible points of hydrolysis.

It was noted that the rate of hydrolysis of the reduced oligosaccharides tended to increase with chain length. A separate experiment was therefore undertaken in which the initial velocities of the hydrolysis of the individual reduced oligosaccharides were determined. Results of this experiment appear in Table 8.

In the hydrolysis of cellotriose, cellobiose was the only product detected (Figure 40a). It was apparent that glucose was not present in sufficient amount to be detected by the PAHBAH reagent. The sensitivity of the reagent towards equimolar amounts of cellobiose and glucose was tested in a separate experiment and it was found that glucose gave a much weaker reaction (25% less) than cellobiose. The reaction product(s) was then analysed on paper chromatography (Figure 41) and it was shown that both cellobiose and glucose were formed from hydrolysis of cellotriose. Standard cellotriose was poorly stained with silver nitrate reagent compared with the other sugars.

Figure 40. Column Chromatography of Cellulodextrins after Hydrolysis by Cellulase III

Column conditions as described for Fig. 39 except that fractions were analysed for reducing sugars only.

(a) Cellotriose hydrolysis: Incubation mixture contained 0.5 ml cellotriose (1 mg/ml, in citrate-phosphate buffer, pH 4.5) and 0.1 ml of enzyme (82.5 µg/ml). Time of hydrolysis at 60°C was 48 h; reaction mixture was then heated for 5 min on a boiling water bath to inactivate the enzyme, cooled and then applied to the column.

(b) Cellotetraose hydrolysis: Incubation mixture contained 0.5 ml cellotetraose, 1 mg/ml as in (a) and 0.1 ml of enzyme (4.12 µg/ml). Time of hydrolysis at 60°C was 1 h; reaction mixture was then treated as in (a).

(c) Cellopentaose hydrolysis: Incubation mixture contained 0.5 ml cellopentaose, 1 mg/ml as in (a) and 0.1 ml of enzyme (2.06 µg/ml). Time of hydrolysis at 60°C was 0.5 h; reaction mixture was then treated as in (a).

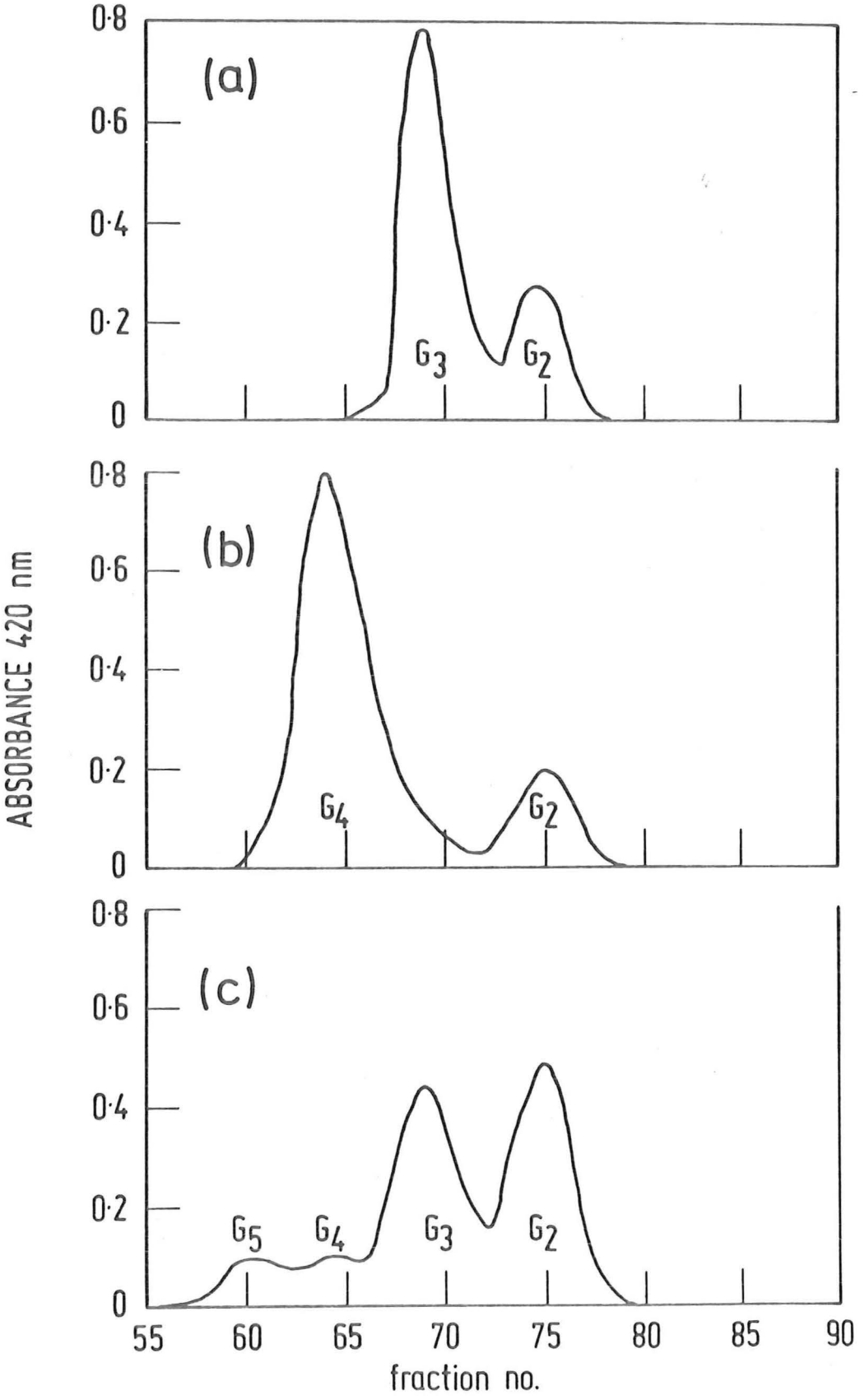

(a)
(b)
(c)
G5
G4
G3
G2
0·8
0·6
0·4
0·2
0
ABSORBANCE 420 nm
55
60
65
70
75
80
85
90
fraction no.

TABLE 8 Relative initial rates of hydrolysis of the reduced cellulodextrins

Compound	Relative Hydrolysis rate
Cellotri-itol	-
Cellotetraitol	1
Cellopentaitol	94
Cellohexaitol	903
Reduced cellulodextrins (D.P. > 7)	1100

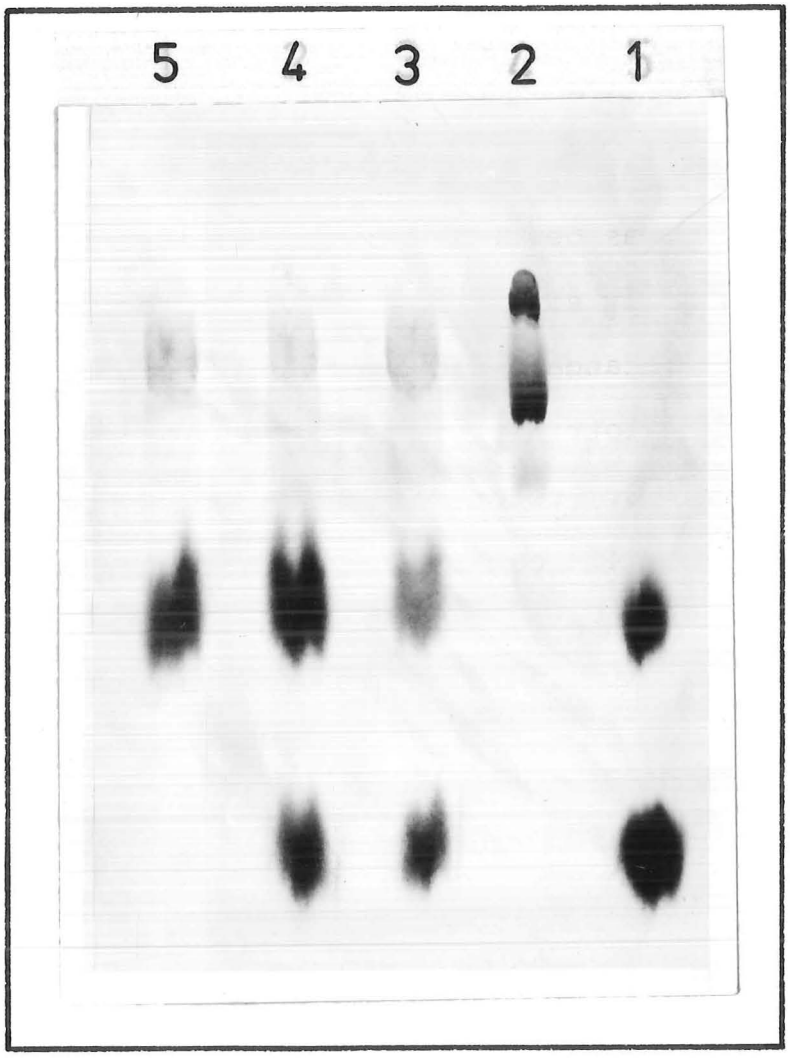
Reaction mixtures contained 2 ml oligosaccharides (1 mg/ml) in citrate-phosphate buffer (0.1 M citric acid; 0.2 M di-basic sodium phosphate), pH 4.5 and 0.4 ml enzyme solution. Incubated at 60°C and 0.3 ml samples were removed at intervals then assayed with 2.5 ml PAHBAH reagent. Enzyme solutions used: for reduced cellotetraitol, 41.2 µg/ml; for cellopentaitol, 4.1 µg/ml and for cellohexaitol and celloheptaitol, 2.0 µg/ml. Total period of incubation was 30 min. except for cello-tetraitol where it was 4 h. Initial velocities were calculated as increase in A_{420} nm/min from the linear portion of the curve. Relative hydrolysis rate was calculated by normalising the different enzyme concentrations. Results are the mean of duplicate determinations.

Cellotetraose was more rapidly cleaved to yield cellobiose (Figure 40b), indicating that hydrolysis of this substrate took place at the middle bond. However, analysis of this hydrolytic product on paper chromatography revealed the presence of cellobiose as well as glucose with the latter present in lesser amount (Figure 41). It is possible that some cellotriose was present in the reaction mixture but was not detected owing to inadequate sensitivity towards this glycosidic trimer.

Hydrolysis of cellopentaose occurred readily with the

Figure 41. Paper Chromatography of the Hydrolytic Products formed from Cellulodextrins by Cellulase III component.

Hydrolysates (0.1 ml) from the hydrolysis of the cellulodextrins as described in Fig. 40 were spotted and the products analysed as described in Materials and Methods. 1. standard glucose and cellobiose; 2. standard cellotriose, cellotetraose and cellopentaose; 3. reaction products from hydrolysis of cellotriose; 4. cellotetraose; and 5. cellopentaose. Concentration of the standard markers used was 1 mg/ml each.


5
4
3
2
1

production of cellotriose and cellobiose in equimolar quantities and trace measure of cellotetraose (Figure 40c). In paper chromatography, only cellobiose was detected (Figure 41).

Quantitative data on the relative rates of hydrolysis of the unmodified cellulodextrins could not be obtained because of the high reducing power inherent with these substrates, which interfered with the PAHBAH reducing sugar assay. It was clear, however, that the rate of degradation increased with chain length.

CHAPTER IV

DISCUSSION

Reports on the ability of *Thermoascus aurantiacus* to degrade cellulose have been contradictory. In a study of the cellulolytic activity of thermophilic fungi and actinomycetes, Fergus (1969) reported that *T. aurantiacus* could not degrade filter paper nor hydrolyse CMC. In contrast, Tansey (1971b), who examined the relative ability of thermophilic fungi species to degrade cellulose using an agar-diffusion technique, had shown that *T. aurantiacus* dissolved the acid-swollen cellulose nearly three times as rapidly as the most vigorous cellulolytic mesophile tested, *Trichoderma viride*. Romanelli *et al.* (1975) also found *T. aurantiacus* to be cellulolytic. Preliminary investigation of a number of thermophiles isolated had confirmed the cellulolytic nature of *T. aurantiacus*. The culture filtrate of this fungus was found to be the most effective in degrading filter paper (Table 1) and CMC (Figure 4). However, the literature lacks information on the hydrolytic breakdown of cellulose or its derivatives by this organism. Thus, the present study was undertaken to further investigate the production of cellulases by *T. aurantiacus* and characterise the enzymes involved.

Before considering the results of this study,

discussion of some general problems connected with the measurement of cellulase activity is in order. The main difficulty encountered was the choice of substrate for enzyme assay. Although there is no doubt that pure cellulose is composed of very long chains of β-1,4-linked glucose units, there is still much controversy on how these chains are arranged in the micro-fibrils and how the latter in turn form the cellulose fibres found in woody materials and which constitute cotton. The question of whether or not regions of less-ordered cellulose chains exist in the microfibril, which is of fundamental importance for the enzymologist, is not yet solved. Consequently a confusing variety of substrates and analytical methods has been employed for the measurement of cellulase activity. It is evident that the whole cellulase complex cannot be characterised by any single method. Some of the more commonly employed substrates are; soluble cellulose derivatives such as CMC, celluloses swollen by either acids or alkali, ball-milled cotton, filter paper strips or powder, and occasionally, relatively undegraded cotton fibres. Estimates of enzymatic activity on these substrates have been based on loss of viscosity (in the case of soluble cellulose derivatives), production of reducing sugars (from both soluble and insoluble celluloses), or weight losses (used with insoluble celluloses).

Endo-glucanase activity can easily be determined by methods based on the reduction of viscosity of a CMC solution. The measurement of β-glucosidase (cellobiase) activity does not create problems either, as the products are easily determined. The measurement of exo-glucanase

activity based on the formation of glucose or reducing sugars from CMC is less clear, because the relative amounts of hydrolysis products vary. The most difficult task, however, is the measurement of the solubilising activity against insoluble cellulose. Unfortunately, this is usually the most interesting and important part of the cellulase complex. Direct assessment of this activity usually involves measurements of loss in tensile strength of strips of cotton duck, loss in weight of cellulosic substances or increase in alkali swelling (swelling factor) of cellulosic materials after incubation with the test organism or its cellulase enzyme. These methods, however, have certain disadvantages. Tests for loss of tensile strength require relatively complicated and costly equipment. Procedures for determining weight loss are very tedious and time consuming, in fact difficult to carry out if large series of analyses or rapid results are needed. The swelling factor method has an advantage in that its action does not depend upon an evaluation of end products and therefore its activity can be measured in the absence of other cellulolytic enzymes. However, the nature of this activity is not understood or even whether its action is on a β-1,4 linkage (Gilligan & Reese, 1954). Walseth activity, measured against Walseth cellulose which represents solid cellulose of low degree of polymerisation and high reactivity has also been used but the difficulty is in obtaining identical batches of substrate. Measurement of cellulase activity therefore presents special problems that do not arise for enzymes hydrolysing soluble substrates.

Furthermore, determination of the activities of cellulolytic enzymes is complicated by two factors. (1) In most cases determinations are not made with purified enzyme but rather in a solution containing a mixture of different cellulolytic enzymes. Because of the synergistic action of these enzymes produced by some organisms, the activity measured is influenced by the proportions of the different enzymes. (2) The substrates used are insoluble macromolecules which are poorly defined as enzyme substrate and this makes standardisation difficult. Also their rate of hydrolysis by enzymes is very low especially with cotton as substrate and a long incubation period is needed. The most convenient substrate is a soluble cellulose derivative, such as CMC. It is rapidly hydrolysed but cannot be used as a universal substrate, since it is not attacked by all of the enzymes present in cellulolytic culture fluids. A cello-oligosaccharide, such as cellotetraose, is attacked by all known types of cellulytic enzymes (Pettersson, 1975), but is rather difficult to prepare.

The assay procedure using filter paper as a cellulose source has proved to be the most satisfactory for routine use in the estimation of hydrolysis of cellulose (Mandels & Weber, 1969; Griffin, 1973; Folan & Coughlan, 1978). Filter paper, though partly degraded and more susceptible to hydrolysis by cellulase than cotton, is considered as highly crystalline and difficult to hydrolyse. Its simplicity and readily duplicated conditions have rendered the filter paper assay being used extensively in

various laboratories to characterise cellulase produced by microorganisms. This method, with minor differences, is also in use in those laboratories most active in achieving cellulose to glucose conversion, e.g., U.S. Army Natick Laboratories, Natick, Northern Regional Research Laboratory, Peoria and Lawrence Berkeley Laboratory, Berkeley. In this work, the filter paper assay measures the amount of reducing sugar expressed as glucose produced from 20 mg Whatman No. 1 filter paper with a reaction time of 24 h. If necessary, dilution of the enzyme used in each assay was made so that the measurement taken was within the linear portion of the standard curve. Direct comparison of the results of this study with those from other workers is difficult, because there are often differences in the choice of cellulose substrate or the experimental conditions used.

Many fungi synthesise enzymes that can degrade soluble cellulosic derivatives. However, the number of fungal species that produce enzymes capable of the extensive degradation of highly ordered (crystalline, insoluble) celluloses to soluble sugars is much less. *T. aurantiacus* produced a complex of cellulase enzymes that enabled it to utilise CMC and filter paper, releasing glucose as the end product. The culture filtrate exhibited both β-glucosidase activity as well as the activities in degrading soluble and insoluble forms of cellulose. Ability of the organism to degrade insoluble cellulose is indicative of C_1 cellulase production (Selby & Maitland, 1967; Whitney *et al.*, 1969).

Growth on solid medium was determined by measurements of the diameters of the colonies because it has been shown to be a reliable method to determine the rate of growth of fungi (Trinci, 1971). This method was used by Evans (1971) to determine the cardinal temperatures for growth of thermophilic fungi isolated from coal spoil tips, by Tansey (1972) in determining the effects of temperature on growth rate of *C. thermophile La Touche* and by Prodromou & Chapman (1974) in studying the effect of nitrogen sources at various temperatures on *Papulaspora thermophile*. The optimum growth temperature on solid medium occurred at 46 - 51°C (Figure 7a) which was in accordance with that reported by Romanelli & co-workers (1975). However, these results differed somewhat from those given by Cooney & Emerson (1964) in their review of the thermophilic fungi. *T. aurantiacus* was listed as giving best growth at 40 - 45°C. The difference observed may be attributed to strain variation and pH of the medium. Growth in liquid medium as measured by cell dry weight (Figure 7b) increased rapidly and reached a maximum of 45°C which was slightly lower than that on solid medium. The surface mycelium in liquid medium was noted as less dense at elevated temperatures. The optimum temperature for cellulase production was recorded as 40°C and reached a plateau after 20 days of incubation (Figure 8). The optimum temperature for enzyme production was lower than that for growth and this is common among some of the thermophilic microorganisms, probably due to thermal instability of the enzymes at elevated temperatures for long incubation periods.

That most, if not all, fungi and bacteria elaborate a mixture of hydrolytic enzymes when utilising cellulose as a nutrient has been demonstrated repeatedly. Most of such data, however, has consisted of electrophoretic or chromatographic separation of cellulases into a number of physically distinguishable components. It has become evident that only by isolation, purification and characterisation of the individual enzymes will the mode of action of cellulases be understood clearly. The main reason why the enzymatic mechanism of cellulose degradation has not yet been completely classified, apart from the lack of well characterised substrates, is undoubtedly the absence of highly purified cellulolytic enzymes. It is extremely difficult to get a cellulolytic enzyme in a state of physico-chemical homogeneity because many components of functionally identical enzymes, characterised by small differences in charge and also often in molecular size, are found (Jermyn, 1962; Eriksson & Pettersson, 1968; Wood & McCrae, 1972). The uncertainty lies in whether the conponents resolved, for instance, by isoelectric focusing actually represent species of the same type of enzyme or if they differ in specificity. Thus conflicting reports of multiplicity and heterogeneity of these enzymes frequently occur in literature (Gascoigne & Gascoigne, 1960; Reese, 1963; Whitaker, 1963; Norkrans, 1967).

The procedures employed for the purification of cellulolytic enzymes in culture filtrates of *T. aurantiacus* are presented in Figure 19. Ammonium sulphate precipitation proved to be an efficient and convenient method in removing a large percentage of the protein whilst retaining most of

the total cellulase activity. This result was a desirable prerequisite for the more selective purification procedures that followed. Low molecular weight substances in the enzyme preparations were removed by chromatography on Bio-Gel P-2. Desalting by dialysis in membranes of cellulose derivatives and filtration through filter paper have been avoided completely because of the strong adsorption of cellulases on cellulose resulted in excessive losses of the enzymes. By chromatography on Sephadex G-100 it was possible to separate β-glucosidase from the cellulolytic enzymes. Partial separation of the cellulase fraction was achieved by the technique of differential adsorption on alkaline-swollen cellulose. The CMC'ase component was more strongly adsorbed than the filter paper degrading activity, but the separation was not complete and elution of the adsorbed enzymes was difficult. Attempts to separate the cellulase components using DEAE-Sephadex eluted with sodium chloride and pH gradients were unsuccessful. However, with batch separation, this problem was not encountered but the proteins were eluted as a single peak. From the isoelectric focusing experiment, it was realised that β-glucosidase and cellulase components have very similar isoelectric points which perhaps explains the difficulty in separating the components using DEAE-Sephadex. Separation of the various enzymic components was finally achieved by preparative disc-gel electrophoresis. The purified enzymes included a β-glucosidase and three cellulase components designated as cellulase I, II and III.

As shown in Table 3, in which the recoveries and total activities of a cellulase (cellulase III) from

T. aurantiacus during the purification procedures are summarised, the specific activity of the purified enzyme represented an approximately 65-fold increase compared with that of the crude extract.

Each of the purified enzymes migrated as a single band in disc-gel electrophoresis (Figure 22) indicating the homogeneity of the components separated. A major band from each of the components in sodium dodecylsulphate gel electrophoresis (Figure 24) was also observed with the exception of cellulase I which showed a number of bands. Minor bands were also evident in the other components, especially in the β-glucosidase fraction. It is possible that the minor bands arose from the main band (i.e. an artifact of the purification scheme). On gel filtration columns used for molecular weight estimation, the purified enzymes migrated as a single symmetrical peak. Isoelectric focusing, however, of the various components (Figure 26) yielded a somewhat different pattern. Cellulase I was again shown as a series of faint bands while both the other two cellulases revealed two major bands. These bands must, therefore, have molecular weights virtually identical with that of the main peak or in the case of cellulase II and III, built of the same subunit. Presumably, the components resolved by isoelectric focusing actually represent species of the same type of enzyme. The isoelectric points of the enzymes with the exception of cellulase I were found to be below 3.2. Higher pI values within the range of 3.8 to 5.0 were recorded for cellulase I. The use of narrow range ampholines (pH 2.0

- 3.0), might have resolved the components further. Wood & McCrae (1972) separated two isoenzymes (pI values 3.80 and 3.95) of the C_1 component of *T. koningii* by electrofocusing in carrier ampholytes covering only 0.5 pH unit (pH 3.72 - 4.25). Eriksson & Pettersson (1975), working with *S. pulverulentum* have described five endocellulases with isoelectric points of 5.32, 4.72, 4.40, 4.65 and 4.20 and molecular weights 32,300, 36,200, 28,300, 37,500 and 37,000 respectively. Small differences in the charge and size of cellulases such as these make them extremely difficult to purify to absolute homogeneity. It has recently been reported that limited proteolysis of cellulase components may be responsible for the multiplicity of enzymes observed in *T. viride* (Nakayama *et al.*, 1976). Conceivably, the diffuse electrophoretic pattern observed with the purified enzymes described here may be the result of very limited proteolysis, such that the charge is altered but there is only a small change in the molecular weight.

Generally, the molecular weight of cellulases is low: ranging from 5,600 (Selby & Maitland, 1965) to 76,000 (Li *et al.*, 1965). The only data on the dimension of such molecules are those of Whitaker *et al.* (1954) who suggested a cigar-shaped molecule with dimensions of about 3.3 x 20 nm. The molecular weights of the purified enzymes from *T. aurantiacus* obtained from gel filtration were calculated to be 85,000 for β-glucosidase; 78,000 for cellulase I; 48,000 for cellulase II and 34,000 for cellulase III. Thus the molecular weight of cellulase I

and II is higher than most of the cellulases reported from other organisms. Cellobiase from *F. solani* with a molecular weight as high as 400,000 has been described by Wood (1971).

Anomalous behaviour of proteins on Sephadex columns, used for molecular weight determination, has been previously reported. Whitaker (1963) concluded that the retardation of lysozyme by Sephadex gels was due to the structural similarities of the gel filtration media and the natural substrate of lysozyme, rather than due to ionic interactions. A similar argument has been proposed for the non-ideal behaviour of amylase (Andrews, 1964). In view of the carbohydrolytic nature of the cellulase, it was perhaps not surprising that the cellulase enzymes were retarded on the Sephadex column. Chromatography on polyacrylamide Bio-Gel was therefore used to eliminate these effects. The fact that the cellulase enzymes were eluted in different volumes (V_e) on Bio-Gel column due to differences in molecular weight and yet always been eluted as a single broad peak from Sephadex columns (Figures 14, 15), is a good indication of a considerable retention of cellulases to Sephadex columns.

Data obtained from sodium dodecylsulphate gel electrophoresis yielded molecular weights of 89,000 for β-glucosidase, 51,000 for cellulase II and 34,500 for cellulase III. Cellulase I was shown as a number of bands with molecular weights ranging from 25,500 to 80,000. These results therefore, indicate that the enzymes β-glucosidase, cellulase II and cellulase III, are single

polypeptide chain devoid of disulphide linkages. The situation with respect to cellulase I is less definite. It is not certain at this stage whether this enzyme is composed of a number of subunits of different molecular weights or whether it is a mixture of different enzymes. The multiplicity and heterogeneity of the enzyme being the result of an artifact of the method employed, the possible action of proteolytic enzymes or the formation of dissociable complexes between enzyme and carbohydrates could not be ruled out.

The cellulases are probably glycoproteins differing however in the extent of their association with carbohydrate (Table 4). The nature of the association of the protein and the carbohydrate has not been determined. The carbohydrate may be covalently linked to the protein moiety in some cases (Okada *et al.*, 1966; Eriksson & Pettersson, 1971), while present as dissociable complexes in others (Wood & Phillips, 1969; Eriksson & Pettersson, 1968). It is not known if the carbohydrate is of importance for the catalytic reaction or merely a residue indicating that the enzyme has been associated with the cell wall. Work done by Jermyn (1955) showed that a β-glucosidase preparation from *Stachybotrys atra* contained a carbohydrate component which was essential for the stability of the enzyme but not for its activity. Complexes between enzyme and polysaccharide have been shown to have caused the apparent heterogeneity observed in some cellulase systems (Jermyn, 1955; Eriksson & Pettersson, 1968): they may also have been the reason for the heterogeneity of *T. koningii* C_1

(Wood & McCraw, 1972), *T. viride* (Berghem & Pettersson, 1973) and *F. sofani* C1 (Wood, 1969). Such a possibility also exists in the case of cellulase I from *T. aurantiacus* which contained the highest carbohydrate content (5.5%) compared with the other two cellulases (2.6 and 1.8% for cellulase II and III respectively).

In general, cellulases characteristically have high temperature optima when compared to other enzyme systems. The cellulases and β-glucosidase described in this work have an optimum temperature of about 70°C (for assay period of 30 min.) which is higher than that of most fungal β-glucanases (50°C). In the case of cellulase II assayed on filter paper for reaction period of 24 h duration, the optimum temperature dropped to 60°C, probably due to inactivation of the enzyme at elevated temperatures at long incubation periods. Cellulase I has the highest optimum temperature of 75°C when assayed on CMC for 30 min.

The high thermostability of the enzymes appears to be a characteristic of fungal cellulase (Mandels & Reese, 1965). It is one of the most important properties of cellulases, since the hydrolysis of cellulose proceeds faster at higher temperatures. Both cellulases and β-glucosidase described here were completely stable up to 65°C for at least an hour. There was little difference observed between the four thermal stability curves. The temperature optima determined in these experiments can be classically described as the balance between the effect of temperature on the rate of reaction and its effect on the rate of enzyme destruction as stated by Dixon & Webb (1964).

The pH optimum of cellulases is generally between pH 4.0 and 6.0. The optimal pH observed for the β-glucosidase and cellulases of *T. aurantiacus* seems consistent with the data observed for other fungi. The enzymes were active in the acid side of neutrality with a sharp optimum on the pH-activity profile (Figure 28) at pH 4.5 to 5.0. The pH stability of the cellulase enzymes (Figure 30) differed from each other with cellulase II showing a remarkably broad pH range of stability from pH 2 to 12 and cellulase III a narrower range of pH 6 to 9. β-glucosidase was recorded as having a pH-stability range of pH 6 to 8.

Kinetic parameters, K_m and v_{max} of the purified enzymes were determined (Table 5). Strictly speaking, K_m is normally determined for enzymic reactions acting on simple, well-defined soluble substrates in which the product(s) formed is known. In the case of highly ordered forms of cellulose such as filter paper, not only is the substrate insoluble and poorly defined but the products of the enzyme reaction are heterogeneous. Moreover, the rate of hydrolysis on different parts of the substrate may vary. In the present work, the K_m values determined for the enzymes hydrolysing filter paper, CMC and yeast-glucan serve to denote the amount of substrate required to achieve half the maximal initial reaction velocity. Little information is available regarding K_m values for cellulose hydrolysis by cellulases. Values of 0.5 and 1.6 mg/ml have been published in studies on CMC hydrolysis by cellulases of *Myrothecium verrucaria* (Halliwell, 1961) and *Trichoderma viride* (Reese & Mandels, 1963), respectively, but the effect of substrate concentration on reaction

rate is complicated by the adsorption of the enzyme onto the substrate (whether it be soluble or insoluble cellulose) in such a way that it is rendered inactive when the ratio of enzyme to substrate is relatively low (Reese & Mandels, 1963). Hurst *et al.*(1977) analysed the pH dependence of K_m and v_{max} of a cellulase from *Aspergillus niger* on CMC. Four pK values between 4.2 and 5.3 were obtained for groups involved in the enzyme substrate complex, indicating the probable importance of carboxyl groups in catalysis. In this work, a K_m value of 4.68 mg/ml was obtained with cellulase III component acting on CMC (DS = 0.65 - 0.85, DP = 3200). It has been shown that the K_m for CMC increases with an increase in degree of substitution (Eriksson & Hollmark, 1969). This probably explained the high K_m value of 19.0 mg/ml calculated by Stutzenberger (1971) for the C_x enzyme hydrolysing a CMC with a degree of substitution of approximately 1.2. Hydrolysis of filter paper by cellulase II was much slower. The K_m for this reaction was recorded as 34.4 mg/ml, corresponding to a v_{max} of 0.8×10^3 units/µg protein. The lowest K_m value of 1.0 mg/ml was obtained with β-glucosidase acting on *p*-nitrophenyl-β-D-glucoside; the turnover number (TN) for this reaction was calculated to be 1.04×10^5. In the case of cellulases, the turnover number was not calculated because of the difference in the substrate used for each enzyme. However, from the activity of cellulase I and III on CMC, it is clear that the turnover number for cellulase III is much greater than that of cellulase I for this substrate.

In the exploration of substrate specificity of the

enzymes, considerable use has been made of the wide range of different β-glucans. In addition, certain soluble derivatives such as CMC and CM-pachyman have been of great value in deciding the action of glucan hydrolases. The use of heterogeneously linked glucans in addition to the homogeneous types has also been useful in distinguishing other types of specific substrate requirements of the enzyme. The availability of linear β-mannans, β-xylan and β-glycol-chitosan with various types of linkages makes it possible to test the effect of alteration of the configuration or size of the monomer unit on the action of various β-glucan hydrolases. To date, few purified enzymes have been tested in this way. An account of the enzyme-substrate relationships among β-glucan hydrolases has been discussed by Barras *et al.*, 1969.

Cellulase I, unlike the other two cellulases, readily hydrolysed the native, mixed β-1,3; β-1,6 polysaccharides such as CM-pachyman, yeast-glucan and laminarin. Lichenan (β-1,4; β-1,3) was degraded by all the cellulase components. Attempts were made to ascertain whether or not the ability to attack β-1,3; β-1,6 linkages was an intrinsic feature of the cellulase I enzyme. The pH-activity profile (Figure 28) as well as the pH and temperature-stability profiles (Figures 29, 30) of the enzyme assayed on CMC (β-1,4 linkage) and yeast-glucan (β-1,3; β-1,6 linkage) were very similar. The difference observed in the temperature optimum on the substrates could be due to inactivation of the enzyme acting on yeast-glucan at high temperature with a long period of incubation

(24 h). On CMC, a much shorter period of incubation (0.5 h) was used. The ability of cellulase II and III in hydrolysing the β-1,4; β-1,3 mixed linked lichenan and not the β-1,3; β-1,6 linked glucans indicates that the 4-β-glucosyl residues are specifically required in the glycosyl portion of the linkage hydrolysed by the enzymes as shown

— G 3 G 4 G 3 G 4 G —

Of the non-glucosidic polymers tested, xylan was the only polysaccharide degraded by cellulase I and β-glucosidase. It has been reported that the cellulase (C_x) and xylanase activities could belong to the same protein as was the case for the highly purified cellulase from *T. viride* isolated by Toda *et al.*(1971): furthermore, endo-cellulase showed endo-xylanase activity whilst exo-cellulase was concomitant with exo-xylanase activity (Shikata *et al.*, 1975). Kanda *et al.*(1976b) have also shown conclusively that xylanase activity is intrinsic to a homogeneous cellulase from *Irpex lacteus*. Such observations have also been reported to be the case of a highly purified β-glucosidase from *Pyricularia oryzae* which split off glucose units one by one from the non-reducing ends of β-glucooligosaccharides, soluble β-1,4 glucans such as CMC, β-1,3 glucan, β-1,6 glucan, and β-1,3; β-1,6 mixed glucan (Hirayama *et al.*, 1978). Hence it is possible that the xylanase activity reported here is an inherent feature of the cellulase and β-glucosidase. Since cellulase I hydrolyses both β-1,4 glucans and β-1,4; β-1,3 glucans its specificity is different from those

enzymes that hydrolyse only mixed glucans. Enzymes from *Bacillus subtilis* have been described that act only on barley glucan or lichenin (Moscatelli *et al.*, 1961; Reese & Perlin, 1963; Reese & Mandels, 1966) indicating a specific requirement for - G 4 G 3 G - in the glycosyl residue of the linkage cleaved. Enzymes from germinating barley have a similar substrate requirement (Chen & Luchsinger, 1964). Corresponding enzymes active mainly on linear β-1,3 glucans have also been reported (Mandels *et al*, 1967; Moore & Stone, 1972; Fleet & Phaff, 1974).

None of the purified enzymes from *T. aurantiacus* was capable of hydrolysing β-1,4-mannan, β-1,4-glycol-chitosan, β-1,4-xylan (except cellulase I and β-glycosidase), or chitin. These substrates contain β-1,4-glycosidic linkage and therefore it is apparent that the enzymes cannot accommodate changes in glucosyl residues to mannosyl, aminoglucosyl, xylosyl, or N-acetyl-D-aminoglucosyl residues. Activity towards sodium polypectate, arabinogalactan and α-1,4 linkage found in polygalacturonic acid was completely absent in all the purified enzyme preparations.

With cellulose as substrate, the cellulase components of *T. aurantiacus* exhibited different properties on different forms of cellulose ranging from native cellulose (cotton yarn) to chemically modified cellulose, CMC. All degraded insoluble cellulose to different extents but the effect of cellulase II was greater than that obtained by the action of the other two cellulases. On the other hand, cellulase II could not hydrolyse the soluble CMC which was

readily degraded by cellulase I and III. However, 94% of the CMC hydrolysing activity was confined to the cellulase III component. None of the cellulase preparations has activity towards either *p*-nitrophenyl-β-D-glucoside or cellobiose. As might be expected, β-glucosidase had no hydrolytic activity towards any form of cellulose tested but degraded cellulodextrin of degree of polymerisation 3 - 6. While it is known that β-glucosidase activity will extend from the dimer to chains of 6- or perhaps even 10-glucose units (Grassman *et al.*, 1933), it is doubtful that its activity would extend to chains of the degree of polymerisation found in CMC (average DP 100+). The ability to break down xylan and other mixed β-1,3; β-1,6 polysaccharides was shown by the purified β-glucosidase reported here. Thus, from the results obtained, it was noted that a distinguishing feature of each of the four enzymic components is a characteristic action on their particular substrate.

Synergism between separated components has been reported for a number of fungal cellulases (Selby & Maitland, 1967; Wood, 1968; Olutiola & Ayres, 1973). The interactions of the purified enzymes have been investigated to gain an understanding of the mechanism of cellulose degradation by *T. aurantiacus*. The resultant hydrolytic products were also analysed. Results shown (Table 6) indicate that there was no synergism between any of the components tested on filter paper. The primary product formed from filter paper by each of the cellulase components acting alone was identified to be cellobiose

(Figure 35a). When acting in the presence of β-glucosidase, glucose was the final product.

From the data obtained, cellulase III was found to be most active in degrading soluble CMC with little or no hydrolytic action on cellobiose. This could represent a form of C_x cellulase, almost completely free of cellobiose activity. Cellulase II was unusual in that it could not break down soluble cellulose but was most active in hydrolysing insoluble cellulose. Thus, it resembles the C_1 component of Olutiola & Ayres (1973) and Selby & Maitland (1967), but differs from the C_1 component of Wood (1968) and Umezurike (1970b) because of its inability to degrade soluble forms of cellulose. Cellulase I was characterised by its broad spectrum of substrate specificity, capable of acting on substrates of β-glycosidic linkages, be it β-1,4 or mixed β-1,3; β-1,6. This enzyme therefore resembles the highly purified cellulase from *T. viride* isolated by Toda *et al.*(1971) and from *Irpex lacteus* by Kanda *et al.*(1976b). Finally, the β-glucosidase component which released glucose from cellobiose but not from cellulosic materials. It is undoubtedly a cellulase-free form of cellobiase.

Based on this information, what must be accounted for now is the attack on both highly ordered and soluble substrates with ultimately the complete conversion to glucose. In proposing a mechanism of cellulolysis, Wood & McCrae (1972) make two statements requiring comment: first, that C_1 is a C_x component unable to attack highly ordered substrates; secondly, they dispute the postulate

of Reese *et al*, (1950) by stating "C_x and not C_1 initiates attack on native cellulose by providing end-groups for C_1." The present work demonstrates that a purveyor of accessible end-groups is not essential for the action of cellulase II component; the enzyme itself is capable both of initiating hydrolysis and of generating end-groups from highly ordered cellulosic substrates without the aid of a C_x type of enzyme (celullase III). Hydrolysis of cellulose by *T. aurantiacus*, therefore, involves a multi-enzymatic process whereby degradation of cellulosic materials of any complexity can be carried out independently by at least one of the cellulase components. Thus, attack on crystalline regions is most efficiently performed by the cellulase II component while cellulase III component catalyses the hydrolysis of soluble cellulose. The elaboration of an enzyme such as cellulase I is perhaps beneficial to the organism in capable of degrading a wide range of substrates. The crucial role of β-glucosidase component in the degradation of native cellulose is to catalyse the hydrolysis of cellobiose to glucose, thereby promoting the action of the cellulase enzymes. Hence, the β-glucosidase component has an important function in the regulating mechanism of enzymatic cellulose degradation.

Cellulase II had no activity on CMC, both in reducing sugars and viscosity assays. Cellobiose was the primary product formed when filter paper was exposed to this enzyme, indicating that cellulase II is an exo-β-1,4-glucan cellobiosyl hydrolase. Work done by Halliwell & Griffin (1973);

Berghem (1974) and Eriksson & Pettersson (1975) lead them to conclude that their C_1 is a cellobiohydrolase. On the other hand, cellulase III component which rapidly decreased the viscosity of a CMC solution with cellobiose and other higher oligosaccharides as products suggested an endo- rather than exo-cellulolytic mode of action. This conclusion was supported by the results obtained from a study on the mode of action by this enzyme against cellulodextrins and reduced cellulodextrins. Therefore, cellulase III is an endo-β-1,4-glucanase. It is interesting to note that while exo-hydrolases for β-1,3- and α-1,4-glucans have been reported from a wide variety of sources, few β-1,4-glucan exo-hydrolases are known. Whether this is because of difficulties in their detection or because of a real scarcity in nature remains to be seen.

Identification of hydrolysis products formed by the action of the cellulase III component on the lower members of the oligosaccharide series and their reduced analogues has enabled a more detailed investigation of the susceptibility to cleavage of the various linkages in the oligosaccharides. The extension of such studies to longer members (CL 7 and upwards) of the homologous series is limited by the availability of substrates owing to the difficulty of fractionation. From the results obtained in the hydrolysis of reduced cellulodextrins, the preferred point of cleavage of the substrates by the enzyme is determined as shown in Scheme 1. The relative rate of hydrolysis of the substrates is shown alongside.

SCHEME 1 Preferred point of cleavage of Reduced Cellulodextrins by Cellulase III Component

		Relative rate
Cellotri-i-tol	G - G $\overset{\downarrow}{-}$ Gol	-
Cellotetraitol	G - G - G $\overset{\downarrow}{-}$ Gol	1
	or	
	G - G $\overset{\downarrow}{-}$ G - Gol	
Cellopentaitol	G - G - G $\overset{\downarrow}{-}$ G - Gol	94
Cellohexaitol	G - G - G $\overset{\downarrow}{-}$ G - G - Gol	903
Reduced cellulodextrin	(DP > 7)	1100

Inspection of the elution profiles (Figures 39a, b, c) shows that the preference for a particular bond was not absolute and that all possible products were formed upon degradation of cellotetraitol, cellopentaitol and cellohexaitol. For example, in the hydrolysis of cellotetraitol as substrate, an appreciable amount of cellobiose was produced indicating that bond 2 was cleaved (bonds are numbered from the non-sorbitol end of the molecule). Although cellotetraose or cellotetraitol were not clearly detected from cellopentaitol, its presence deduced from the fact that glucose was obtained, indicated bond 1 and 4 were also cleaved in addition to bond 2 and 3. With cellohexaitol as substrate , the detection of all possible

products showed that all the bonds were cleaved. The relative amounts of the products indicate, however, that the central linkages are the preferred points of cleavage. This clearly demonstrates the departure from the classical interpretation of "random" hydrolysis (i.e. the equal susceptibility of all bonds to cleavage). The observed preference for non-terminal linkages confirms the endo-action of the enzyme which was previously indicated by the demonstration that the cellulase rapidly reduces the viscosity of a CMC solution (Figure 38) with the simultaneous slow release of reducing sugars (Figure 36).

The rate of hydrolysis increased with chain length of the reduced cellulodextrins (Table 7). The relative rate increased about ninety-fold going from cellotetraitol to cellopentaitol, while there was a slight increase of about ten-fold going from cellopentaitol to cellohexaitol. The same trend in the relative rates for the reduced cellulodextrins has been observed earlier by Hurst *et al* (1978) working with a cellulase from *A. niger*. The increase was not dramatic in going from cellohexaitol to reduced cellulodextrins (DP > 7). Based on these observations, it may be concluded that in the specificity region of the enzyme there are at least five or possibly six sub-sites in the active centre that recognise or bind glucose residues. These observations may be compared with the work done on other cellulases in which the K_m for the hydrolysis of the β-1,4-oligosaccharides is also dependent on chain length. The decrease in K_m with increasing chain length of the oligosaccharides has been observed by Li *et al*.(1965)

and they reported that the optimum substrate chain length was at least six glucosyl units long. Pettersson (1969) working on a cellulase from *Penicillium notatum*, has concluded that the specificity region of the enzyme was five glucose units in length. Similarly, Whitaker (1954) and Hanstein & Whitaker (1963) showed that the specificity region of the *Myrothecium* cellulase was at least five glucose units in length. The decrease in K_m with increasing chain length of the oligoglucosides might reflect an increasing tendency to form enzyme substrate complexes. It has been assumed that the sorbitol residue does not alter the action of the enzyme and also that it has the same steric influence as a glucose unit. This was shown to be the case by Pettersson (1969) when he noted that the addition of a β-sorbitol residue to cellotetraose has about the same effect on K_m as the addition of a β-glucosyl residue.

In the hydrolysis of unmodified cellulodextrins, basically the same pattern in the preferred point of cleavage compared with that of the reduced cellulodextrin was obtained and is shown in Scheme 2.

SCHEME 2 Deduced sites of hydrolysis of cellulodextrins by cellulase III component

Cellotriose	G - G ↓ G
Cellotetraose	G - G ↓ G - G
Cellopentaose	G - G - G ↓ G - G
	or
	G - G ↓ G - G - G

Thus, hydrolysis of cellotriose yielded cellobiose and glucose as products: glucose was detected by paper chromatrography stained with silver nitrate reagent. However, cellotetraose was cleaved to give predominantly cellobiose although trace amount of glucose was also detected on paper chromatography. This result clearly indicated that hydrolysis of this substrate took place at the middle bond in contrast to bond 3 which was the preferred site of cleavage in cellotetraitol. To account for this difference, it must be concluded that with a small chain length substrate such as cellotetraose, it does appear that the sorbitol residue has an effect on the action of the enzyme. Such an effect might be minimal in the case of cellopentaose in which the major products, cellotriose and cellobiose in approximately equimolar amounts, were obtained. Although quantitative data on the relative rate of hydrolysis of the native cellulodextrins could not be obtained, it was clear that the rate of degradation increased with chain length.

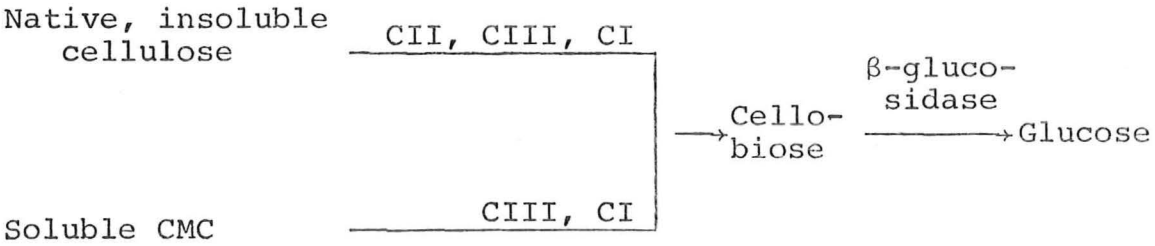
In light of the experiment with the cellulodextrins, it is possible to explain the relatively slower rate of hydrolysis of highly ordered forms of cellulose by the cellulase III component. The enzyme was found to degrade cellotriose very slowly. Thereafter, the rate of degradation increased rapidly with the degree of polymerisation of the oligosaccharide chain up to a limit of six glucose residues. In crystalline cellulose the polysaccharide chains are very closely packed and it is improbable that longer segments of polysaccharide chains are accessible to

the enzyme. It is also of interest to note that after degradation of highly ordered cellulose (filter paper) for 48 h, the molar ratio of glucose to cellobiose (estimated visually) on paper chromatography, was approximately 1:1 (Figure 35b), which is the ratio expected upon degradation of cellotriose. The acid swollen cellulose gave glucose and cellobiose approximately in the molar ratio 1:2 (Figure 35c) which is the expected ratio upon degradation of longer oligosaccharides. These results support the hypothesis that at least in the early stage of cellulose degradation only short segments of polysaccharide chains in unmodified insoluble cellulose are accessible to the enzyme.

It can be concluded that the cellulase III component functions as an endo-glucanase and should be designated as β-1,4-glucan 4-glucanohydrolase (EC 3.2.1.4). It should be pointed out that the typical preference for the cleavage of nonterminal bonds in the short chain oligosaccharides does not necessarily rule out the possibility that longer chains are cleaved in an approximately random manner. Although in most cases the properties of the substrate (substitution or accessibility of linkages) rather than the intrinsic properties of the enzyme, determine the method of degradation.

Until now, the mechanism of enzymatic cellulose degradation is not completely understood although it had been the subject of intensive investigation. Conflicting reports concerning the heterogeneity and multiplicity of cellulolytic enzymes are frequently published. At the

present state of knowledge, it is premature to carry out a comparison of cellulases from different microorganisms with the intention of finding a unifying concept and a model for the enzymatic mechanism which is common to all cellulolytic microorganisms. The various hypotheses suggested may all be acceptable and may at least in part represent alternative mechanisms functioning in different microorganisms. In the case of *T. aurantiacus*, degradation of the native, insoluble and soluble celluloses by purified enzymes of this organism is as shown:



Degradation of the native, insoluble cellulose such as filter paper is most efficiently carried out by the cellulase II component while cellulase III catalyses the hydrolysis of soluble CMC. Cellobiose was the primary product formed in both cases. β-glucosidase completes the reaction by converting cellobiose to glucose. It should be emphasised that the three cellulases act independently.

As there is a great amount of research activity going on in this field it is likely that new types of cellulolytic enzymes will be discovered in the future. It must also be borne in mind that some cellulolytic microorganisms might have evolved pathways that cannot yet

be expressed solely in terms of our limited knowledge of a synergistic reaction involving C_1 and component C_x.

So far, attempts to utilise cellulose as a commercial fermentation substrate have been disappointing. The low rate of hydrolysis by mesophilic microorganisms has been cited as one of the chief obstacles to microbiological conversion (Bellamy, 1969). The possibility of a high rate of cellulose degradation by thermophilic fungi, as a result of their rapid metabolic rates, makes their study particularly attractive and suggests application of these fungi for industrial production of cellulases. In addition, high incubation temperatures used in their cultivation would greatly limit the number of contaminants able to grow, allowing the use of relatively unsophisticated equipment for large-scale fermentation.

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APPENDIX

Solid Media

YG : Yeast-glucose agar (Emerson, R., 1941)

Difco powdered yeast extract	5.0 g
Glucose	10.0 g
Agar	20.0 g
Distilled water	1000 ml
pH adjusted to 5.0	

YpSs : Yeast-starch agar (Emerson, R., 1941)

Difco powdered yeast extract	4.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Soluble starch	15.0 g
Agar	20.0 g
Distilled water	1000 g
pH adjusted to 5.0	

Standard 10.0 x 2.0 cm glass or 9.0 x 1.5 cm presterilised plastic petri dish containing 30 ml of culture medium were used.

Unless otherwise stated, the culture temperature on solid media was 50°C.

Liquid Media

Yeast-glucose medium

Difco powdered yeast extract	5.0 g
Glucose	10.0 g
Distilled water	1000 ml
pH adjusted to 5.0	

Reese-Mandels (1963) medium

K_2HPO_4	1.0 g
$(NH_4)_2HPO_4$	1.2 g
Urea	1.4 g
$MgSO_4.7H_2O$	0.3 g
Difco powdered yeast extract	0.1 g
Difco Bacto peptone	1.0 g
Carboxymethyl-cellulose	10.0 g
Microelement solution (Fergus, 1964)	1.0 ml
Distilled water	1000 ml
pH adjusted to 5.0	

Microelement stock solution (Fergus, 1964)

1.0 ml contained

$Fe(NH_4)_2(SO_4)_2.6H_2O$	1.0 mg
$ZnSO_4$	1.0 mg
$MnSO_4.4H_2O$	0.5 mg
$CaSO_4.5H_2O$	0.08 mg
$CoCl_2$	0.07 mg
H_3BO_3	0.10 mg

Fergus (1969) medium

K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.3 g
Difco Bacto peptone	1.0 g
Difco powdered yeast extract	0.1 g
Microelement solution (Fergus, 1964)	1.0 ml
Distilled water	1000 ml
Filter paper (1.0 cm^2)	2.0 g per 60 ml medium

pH adjusted to 6.5

All media were sterilised by autoclaving for 20 min. at 121°C.